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METHOD FOR IDENTIFYING INHIBITORS OF G PROTEIN COUPLED RECEPTOR SIGNALING

BACKGROUND OF THE INVENTION

1. Technical Field

[0001] The present invention generally pertains to the field of modulating G protein-coupled receptors (GPCR) and of identifying and preparing G protein coupled receptor inhibiting compounds.

2. Description of the Background Art

[0002] A great number of chemical messengers exert their effects on cells by binding to G protein-coupled receptors. Ligand binding to those receptors is transduced by heterotrimeric G proteins into intracellular responses. Four main classes of G proteins are distinguishable: Gs, Gi, Gq and G12. G protein-coupled receptors (GPCR) include a wide range of biologically active receptors, such as hormone receptors, viral receptors, growth factor receptors, chemokine receptors, sensory receptors and neuroreceptors. These receptors are activated by the binding of ligand to an extracellular binding site and mediate their actions through the various G proteins. The molecular interactions that occur between the receptor and the G protein are fundamental to the transduction of environmental signals into specific cellular responses. The G proteins themselves play important roles in determining the specificity and temporal characteristics of the cellular response to the ligand-binding signal.

[0003] In the inactive state, G proteins are heterotrimeric, consisting of one α, one β and one γ subunit, and a bound deoxyguanosine diphosphate (GDP). Receptor-catalyzed guanine nucleotide exchange resulting in deoxyguanosine triphosphate (GTP) binding to the α subunit activates the G protein. Gα-GTP dissociates from the Gβγ subunits, allowing the Gβγ dimer and the Gα-GTP subunit each to activate downstream effectors. Hydrolysis of GTP to GDP deactivates the complex and turns off the cellular response.

[0004] G protein-coupled receptors have seven transmembrane helices which form, on the intracellular side of the membrane, the G protein binding domain. Experiments have suggested that activation of the receptor by ligand binding changes conformation of the receptor, unmasking G protein binding sites on the intracellular face of the receptor. The heterotrimeric G protein interacts with GPCR in a multi-site fashion with the major site of contact between them at the carboxyl terminus of the Gα subunit. Hamm et al., *Science* 241:832-5, 1998; Osawa and Weiss, *J. Biol. Chem.* 270:31052-8, 1995; Garcia et al., *EMBO J.* 14:4460-9, 1995; Sullivan et al., *J. Biol. Chem.* 269:21519-21525, 1994; West et al., *J. Biol. Chem.* 260:14428-30, 1985.

[0005] The carboxyl terminal 11 amino acids are most important to receptor interaction and to the specificity of this interaction, Martin et al., *J. Biol. Chem.* 271:361-366, 1996; Kostenis et al., *Biochemistry* 36:1487-1495, 1997, however other regions on Gα also are involved in receptor contact. In addition, portions of the Gβγ dimer have been implicated in GPCR binding. See Onrust et al., *Science* 275:381-384, 1997; Lichtarge et al., *Proc. Natl. Acad. Sci. USA* 93:7507-7611, 1996; Mazzoni and Hamm, *J. Biol. Chem.*

271:30034-30040, 1996; Bae et al., *J. Biol. Chem.* 272:32071-32077, 1997. The carboxyl terminal amino acid regions of Gα proteins (and other GPCR binding regions of the heterotrimeric G protein) not only provide the molecular basis of receptor-mediated activation of G proteins, but they also play an important role in determining the fidelity of receptor activation. Conklin et al., *Nature* 363:274-276, 1993; Conklin et al., *Mol. Pharmacol.* 50:885-890, 1996.

[0006] The G-protein complex thus serves a complex role, as an intermediate that relays the signal from receptor to one or more specific effectors, and as a clock that controls the duration of the signal. Hamm and Gilchrist, *Curr. Opin. Cell Biol.* 8:189-196, 1996. Multiple receptors can activate a single G protein subtype, and in some cases a single receptor can activate more than one G protein, thereby mediating multiple intracellular signals. In other cases, however, interaction of a receptor with a G protein is regulated in a highly selective manner such that only a particular heterotrimer is bound.

[0007] Because G proteins and their receptors influence a large number of intracellular signals mediated by a large number of different chemical ligands, considerable potential for modulation of disease pathology exists. Many medically significant biological processes are influenced by G protein signal transduction pathways and their downstream effector molecules. See Holler et al., *Cell. Mol. Life Sci.* 340:1012-20, 1999. Therefore, G protein-coupled receptors and their ligands are the target for many pharmaceutical products and are the focus of intense drug discovery efforts. Over the past 15 years, nearly 350 therapeutic agents targeting GPCRs have been successfully introduced

into the market. Because of the ubiquitous nature of G protein-mediated signaling systems, and their influence on a great number of pathologic states, it is highly desirable to find new methods of modulating these systems.

[0008] Most currently available drugs affecting GPCRs act by antagonizing the binding between a G protein-coupled receptor and its extracellular ligand(s). On the other hand, receptor subtype-selective drugs have been difficult to obtain. A drawback to the classical approach of designing drugs to interfere with ligand binding has been that conventional antagonists are ineffective for some GPCRs such as proteinase activated receptors (PAR) due to the unique mechanism of enzymatic cleavage of the receptor and generation of a tethered ligand. In other cases, intrinsic or constitutive activity of receptors leads to pathology directly, thus rendering antagonism of ligand binding moot. For these reasons, alternative targets for blocking the consequences of GPCR activation and signaling are highly desirable.

[0009] One potential alternative target for inhibition by new pharmaceuticals has been the receptor-G protein interface on the interior of the plasma membrane. Konig et al., *Proc. Natl. Acad. Sci. USA* 86:6878-82, 1989; Acharya et al., *J. Biol. Chem.* 272:651924, 1997; Verrall et al., *J. Biol. Chem.* 272:6898-902, 1997. The carboxyl terminus of Gα and other regions of the G protein heterotrimer conform to a binding site at the cytoplasmic face of the receptor. Sondek et al., *Nature* 372:276-9, 1994; Lambright et al., *Nature* 369:621-8, 1994; Lambright et al., *Nature* 379:311-9, 1996; Sondek et al., *Nature* 379:369-74, 1996; Wall et al., *Science* 269:1405-12, 1996; Mixon et al., *Science* 270:954-

960, 1995. Peptides corresponding to these binding regions or mimicking these regions, can block receptor signaling or stabilize the active agonist-bound conformation of the receptor. Hamm et al., *Science* 241:832-5, 1988; Gilchrist et al., *J. Biol. Chem.* 273:14912-9, 1998. For example, in the case of rhodopsin, the rod photoreceptor, the Gα C-terminal peptide, Gα 340-350, stabilizes the receptor in its active metarhodopsin II conformation. Hamm et al., *Science* 241-832-5, 1988; Osawa and Weiss, *J. Biol. Chem.* 270:31052-31058, 1995. Similarly, two carboxyl terminal peptides from GαS (354-372 and 384-394), but not the corresponding peptides from $G\alpha i_2$, evoke high affinity agonist binding to β_2-adrenergic receptors and inhibit their ability to activate Gαs and adenylyl cyclase. Rasenick et al., *J. Biol. Chem.* 269:21519-21525, 1994.

[0010] In general, GPCRs require agonist binding for activation. However, modifications to the receptor amino acid sequence can stabilize the active state conformation without the requirement for a ligand. Stabilization by such ligand-independent means is termed "constitutive receptor activation." Constitutive (or agonist-independent) signaling activity in mutant receptors has been well documented, but only a few GPCRs have been shown to exhibit agonist-independent activity in the wild type (or native) form. For example, native dopamine D1B and prostaglandin EP1b receptors possess constitutive activity (Tiberi and Caron, J. Biol. Chem. 269:27925-27931, 1994; Hasegawa et al., J. Biol. Chem. 271:1857-1860, 1996). A number of GPCRs, for example, receptors for thyroid-stimulating hormone (Vassart et al., Ann. N.Y. Acad. Sci. 766:23-30, 1995), causing disease in humans have been found to be

mutated to exhibit agonist-independent activity. Experimentally, several single amino acid mutations have produced agonist independent activity. ß2 and α2 adrenergic receptors, for example, mutated at single sites in the third cytoplasmic loop show constitutive activity (Ren et al., J. Biol. Chem. 268:16483-16487, 1993; Samama et al., Mol. Pharmacol. 45:390-394, 1994). In some cases, a large deletion mutation in the carboxy tail or in the intracellular loops of GPCRs has led to constitutive activity. For example, in the thyrotropin releasing hormone receptor a truncation deletion of the carboxyl terminus Nussenzveig et al., J. Biol. Chem. 268:2389-2392, 1993; Matus-Leibovitch et al., J. Biol. Chem. 270:1041-1047, 1995 or a smaller deletion in the second extracellular loop of the thrombin receptor (Nanevicz et al., J. Biol. Chem. 270:21619-21625, 1995) renders the receptor constitutively active.

[0011] These finding have led to a modification of traditional receptor theory (Samama et al., J. Biol. Chem. 268:4625-4636, 1993). It is now thought that receptors can exist in at least two conformations, an inactive conformation (R) and an activated conformation (R*), and that an equilibrium exists between these two states that markedly favors R over R* in the majority of receptors. It has been proposed that in some native receptors and in the mutants described above, there is a shift in equilibrium in the absence of agonist that allows a sufficient number of receptors to be in the active R* state to initiate signaling.

[0012] Negative antagonism is demonstrated when a drug binds to a receptor that exhibits constitutive activity and

reduces this activity. Negative antagonists appear to act by constraining receptors in an inactive state (Samama et al., Mol. Pharmacol. 45:390-394, 1994). Although first described in other receptor systems (Schutz and Freissmuth, J. Biol. Chem. 267:8200-8206, 1992), negative antagonism has been shown to occur with GPCRs such as opioid (Costa and Herz, Proc. Natl. Acad. Sci. USA 86:7321-7325, 1989; Costa et al., Mol. Pharmacol. 41:549-560, 1992), ß2-adrenergic (Samama et al., Mol. Pharmacol. 45:390-394, 1994; Pei et al., Proc. Natl. Acad. Sci. USA 91:2699-2702, 1994; Chidiac et al., Mol. Pharmacol. 45:490-499, 1994), serotonin type 2C (Barker et al., J. Biol. Chem. 269:11687-11690, 1994), bradykinin (Leeb-Lundberg et al., J. Biol. Chem. 269:25970-25973, 1994), and D1B dopamine (Tiberi and Caron, J. Biol. Chem. 269:27925-27931, 1994) receptors. That being stated, the concept of a constitutively active receptors offer insights which explain pathophysiologic conditions. For example, a constitutively active receptor in a disease process such as hypertension may no longer be under the influence of the sympathetic nervous system. In hypertension, a constitutively active GPCR may be expressed in any number of areas including the brain, kidneys or peripheral blood vessels. A newly recognized class of drugs (negative antagonists or inverse agonists) which reduce undesirable constitutive activity can act as important new therapeutic agents. Thus, a technology for identifying negative antagonists of both native and mutated GPCRs has important predictable as well as not yet realized pharmaceutical applications. Furthermore, because constitutively active GPCRs are tumorigenic, the identification of negative antagonists for these GPCRs can

lead to the development of anti-tumor and/or anti-cell proliferation drugs.

[0013] Mutagenesis of this same region of Gαt has identified several specific amino acid residues in this binding region crucial for Gαt activation by rhodopsin. Martin et al., *J. Biol. Chem.* 271:361-6, 1996. Substitution of three to five carboxyl-terminal amino acids from Gαq with corresponding residues from Gαi allowed receptors which signal exclusively through Gαi subunits to activate the chimeric α subunits and stimulate the Gαq effector, phospholipase C β. Conklin et al., *Nature* 363:274-276, 1993; Conklin et al., *Mol. Pharmacol.* 50:885-890, 1996. All of these studies suggest that Gα carboxyl peptide sequences are responsible for the specificity of the signaling responses of the individual G proteins. There are 16 unique Gα subunits ($G\alpha i_1$, $G\alpha i_2$, $G\alpha i_3$, $G\alpha O_1$, $G\alpha O_2$, GαZ, Gαt, Gαq, Gα11, Gα14, Gα5, Gα12, Gα13, Gα15/16, GαOIF and Gαgust) thought to mediate specific interaction with different GPCRs, several hundred of which have been cloned. Thus, peptides corresponding to G protein regions which bind the GPCR could be used as competitive inhibitors of receptor-G protein interactions. Hamm et al., *Science* 241-832-5, 1988; Gilchrist et al., *J. Biol. Chem.* 273-14912-9, 1998. Drug discovery approaches which take advantage of this opportunity, however, are not available. Jones et al., Expert Opin. Ther. Patents 9(12): 1641, 1999.

[0014] An important aspect of the modern drug discovery process is the identification of potent lead compounds for use in modern high throughput screening assays. One of the major challenges confronting companies using high throughput screening is the difficulty of identifying useful lead

compounds from very large combinatorial libraries. When literally hundreds of thousands of compounds are screened, characterizing the compounds which test positive (including false positives) is an expensive and time-consuming process. Hence, a method which can identify potent lead compounds and reduce the number of false positives in the screening process would be very desirable.

SUMMARY OF THE INVENTION

[0015] This invention provides a method of identifying a G protein coupled receptor signaling inhibitor, which comprises (a) providing a peptide library based on a native G protein coupled receptor binding peptide; (b) screening said peptide library for high affinity binding to said G protein coupled receptor; (c) selecting a member of said peptide library having binding to said G protein coupled receptor of higher affinity than that of the native peptide; (d) providing a library of candidate compounds to screen for binding to said G protein coupled receptor; (e) screening said library of candidate compounds for high affinity binding to said G protein coupled receptor in competition with a member of said peptide library selected in step (c); and (f) identifying a member of said library of candidate compounds having binding to said G protein coupled receptor of equal or higher affinity than that of the peptide selected in step (c).

[00016] The invention also provides, in a further embodiment, an enzyme-linked immunosorbant assay which comprises the steps of (a) immobilizing a G protein coupled receptor onto a solid support; (b) providing a protein-peptide fusion protein display library; (c) incubating

members of said protein-peptide fusion protein display library with said immobilized G protein coupled receptor in the presence of said G protein coupled receptor binding peptide under conditions such that members of protein-peptide fusion protein display library having a binding affinity for said G protein coupled receptor at least as high as said G protein coupled receptor binding peptide bind to said immobilized G protein coupled receptor; (d) removing unbound members of said protein-peptide fusion protein display library; (e) incubating said bound protein-peptide fusion protein display library with antibodies which specifically recognize the protein portion of said protein-peptide fusion protein display library members under conditions such that said antibodies specifically bind to said protein-peptide fusion protein display library members; (f) removing unbound antibodies; and (g) detecting said bound antibodies.

[0017] In yet a further embodiment, the invention provides a method of identifying a G protein coupled receptor signaling inhibiting peptide, which comprises (a) providing a peptide library based on a native G protein coupled receptor binding peptide; (b) screening said peptide library for high affinity binding to said G protein coupled receptor; and (c) selecting a member of said peptide library having binding to said G protein coupled receptor of higher affinity than that of the native peptide.

[0018] In yet a further embodiment, the invention provides a method of identifying a G protein coupled receptor signaling inhibitor compound, which comprises (a) providing a library of candidate compounds to screen for binding to said G protein coupled receptor; (b) providing a

high affinity G protein coupled receptor binding peptide; (c) screening said library of candidate compounds for high affinity binding to said G protein coupled receptor in competition with said high affinity G protein coupled receptor binding peptide; and (d) identifying a member of said library of candidate compounds having binding to said G protein coupled receptor of equal or higher affinity than that of the peptides of step (b).

[0019] In yet a further embodiment, the invention provides a method of inhibiting G protein coupled receptor signaling which comprises contacting a compound with said G protein coupled receptor which interferes with binding of said G protein coupled receptor to its cognate G proteins.

[0020] The invention provides, in yet a further embodiment, a compound selected from the group consisting of SEQ ID NOS:14, 16, 20, 22, 26, 28, 30, 32, 34, 36, 38, 40, 42, 46-105, 115-132 and 147-305.

[0021] In yet a further embodiment, the invention provides a method for providing a therapeutic G protein coupled receptor signaling modifier peptide to a mammal which comprises administering to said mammal an expression construct which expresses a peptide according to SEQ ID NOS:14, 16, 20, 22, 26, 28, 30, 32, 34, 36, 38, 40, 42, 46-105, 115-132 and 147-305.

BRIEF DESCRIPTION OF THE DRAWINGS

[0022] Figure 1 is a schematic diagram showing the basis for the affinity screening method used to separate and identify GPCR binding peptides.

[0023] Figure 2 is a schematic diagram of vector pJS142.

[0024] Figure 3 is a schematic diagram showing an ELISA procedure.

[0025] Figure 4 provides binding data for LacI peptide fusion proteins to PAR1 receptor. pELM6 is the MBP vector alone; pELM17 is the MBP-native Gt340-350 peptide fusion protein.

[0026] Figure 5 is a bar graph comparing binding of high affinity clones to the clone of peptide 8.

[0027] Figure 6 is a bar graph presenting results of a competitive binding assay identifying high affinity rhodopsin binding peptides.

[0028] Figure 7 is a bar graph showing competitive inhibition of high affinity peptides to rhodopsin by heterotrimenic Gt.

[0029] Figure 8 presents ELISA results from panning CHO cells overexpressing human thrombin receptor (PAR1) using purified MBP-C-terminal fusion proteins. MBP-G11 = xxxx (SEQ ID NO: 1) LQLNLKEYNLV (SEQ ID NO: 2); PAR-13 = VRPS (SEQ ID NO: 3) LQLNRNEYYLV (SEQ ID NO: 4); PAR-23 = LSRS (SEQ ID NO: 5) LQQKLKEYSLV (SEQ ID NO:6); PAR-33 = LSTN (SEQ ID NO: 7) LHLNLKEYNLV (SEQ ID NO: 8); PAR-34 = LPQM (SEQ ID NO: 9) QRLNVGEYNLV (SEQ ID NO: 10); PAR-45 = SRHT (SEQ ID NO: 11) LRLNGKELNLV (SEQ ID NO:194).

[0030] Figure 9 presents a dose-response curve of SF9 membranes (PAR1 receptor) assayed with lacI-Gq lysates.

[0031] Figure 10 is a concentration response curve demonstrating binding of native Gq peptide-maltose binding protein to PAR1 reconstituted in lipid vesicles.

[0032] Figure 11 is a schematic diagram showing an exemplary cDNA minigene construct.

[0033] Figure 12 is an agarose gel of a NcoI digest of minigene vector. Lane 1 is a 1 kb DNA ladder; lane 2 is pcDNA 3.1; lane 3 is pcDNA-Gαi; lane 4 is pcDNA-GαiR; and lane 5 is pcDNA-Gαq.

[0034] Figure 13 is an agarose gel of PCR products showing transcription of peptide minigene RNA in transfected cells. Lane 1 contains size markers, lane 2 contains PCR products from cells transfected with pcDNA-GiR, lane 3 contains PCR products from cells transfected with pcDNA-Gi, and lane 4 contains PCR products from cells transfected with pcDNA3.1, the empty vector.

[0035] Figure 14 is a bar graph showing the relative [^{3}H] inositol phosphate production after thrombin stimulation normalized against the basal value.

[0036] Figure 15 presents data showing GPCR binding peptide inhibition of intracellular calcium concentration increases. Figure 15A presents fluorescence ([Ca^{++}]; level) increase 30 seconds after thrombin addition. Figure 15B shows the kinetics of [Ca^{++}] fluorescence changes after cell stimulation with thrombin.

[0037] Figure 16 presents data showing GPCR binding peptide inhibition of thrombin-induced phosphoinositol (P1) hydrolysis.

[0038] Figure 17 is a bar graph indicating relative thrombin-mediated fold increases of MAPK activity in cells expressing GPCR-binding peptides.

[0039] Figure 18 shows reduction of thrombin-induced transendothelial electrical resistance in cells expressing Gαq, Gαi, GαiR or empty vector.

[0040] Figure 19 is a series of photographs of cells stained for F-actin, showing the inhibition of stress fiber formation after exposure to thrombin in cells expressing pcDNA-G12 or pcDNA-G13 minigene construct.

[0041] Figure 20 presents data showing blockade of M_2 mAChR response by Gαi peptide expression.

[0042] Figure 21 demonstrates selective G protein mediated adenylyl cyclase inhibition in cells expressing minigene constructs containing Gα carboxyl terminal peptide inserts.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0043] The present invention involves a method of identifying compounds which can interfere with binding at the interface between a G protein-coupled receptor (GPCR) and its cognate G proteins. These compounds inhibit G protein-mediated signaling and thus can be used as pharmaceuticals, as lead compounds for identification of potential useful drugs, and as components of assays which identify drug candidates. Methods for screening and drug identification use peptides that mimic the structure of the GPCR binding regions of G proteins and are able to inhibit receptor-G protein interactions specifically and with high affinity. These high affinity peptides can be delivered into cells in the context of an expression construct to act as blockers of specific receptor-mediated cellular responses *in vitro* and *in vivo* or can be administered directly to a patient. The peptides also form the basis of a screening, identification and selection process to provide traditional

pharmaceutical compounds. In particular, the invention allows one to identify high affinity analog peptides that block the receptor-G protein interface for a particular G protein and to use these high affinity analogs in a high throughput screen to identify other peptides or small molecules that likewise specifically antagonize GPCR signaling for a G protein or class of G proteins.

[0044] Small molecules can be used in an analogous high throughput screening process to identify further compounds. "Small molecule" denotes any non-peptide organic compound which binds or interferes with binding to the interfacial region of a GPCR or is a candidate for such action. These peptides or small molecules directed at the receptor-G protein interface can be designed using the inventive method to inhibit biological processes that employ signaling through a GPCR. This approach is useful in targeting G protein-GPCR interactions for which there are no available antagonist ligands, orphan receptors the ligands of which are not known, mutant constitutively activated receptors, antibody-crosslinked irreversibly activated receptors such as TSH receptors in Graves Disease, and proteinase activated receptors (PAR). It works equally well, however, with any GPCR-G protein interaction and more broadly, with receptor-protein interactions in general.

[0045] Because the method is useful for identifying high affinity compounds that can antagonize virtually any GPCR, the approach is useful in identifying compounds which can prevent, ameliorate or correct dysfunctions or diseases in which a specific class of G proteins is relevant. Conditions and disease states for which this method is useful include, but are not limited to: stroke; myocardial

infarction; restenosis; atherosclerosis; hypotension; hypertension; angina pectoris; acute heart failure; cardiomyocyte apoptosis; cancers; infections such as bacterial, fungal, protozoan and viral infections, and particularly infections caused by HIV-1 or HIV-2; septic shock; pain; chronic allergic disorders; asthma; inflammatory bowel disease; osteoporosis; rheumatoid arthritis; Graves disease; post-operative ileus; urinary retention; testotoxicosis; ulcers; obesity; benign prostatic hypertrophy; and psychotic and neurological disorders including anxiety, epilepsy, schizophrenia, manic depression, Parkinson's disease, Alzheimer's disease, delirium, dementia, drug addiction, anorexia, bulimia, mood disorders and sleep disorders; smoking cessation and any other disease or condition that can be treated by G protein coupled receptor inhibition. Treatment of this diverse set of disorders is possible because the receptors to which various G proteins bind differ enough to allow the creation of a battery of analog peptides which can specifically interface with different GPCR or different classes or groups of GPCR.

[0046] With the inventive screening methods, the sequences identified in a particular screen do not bind to all receptors, but only to the particular receptor of interest. The interaction between a G protein and a GPCR is quite specific. For example, a difference in one amino acid can substantially reduce or eliminate the ability of the $G\alpha i_{1/2}$ peptide to bind the A1 adenosine G protein coupled receptor-G protein interface. Gilchrist et al., *J. Biol. Chem.* 273:14912-14919, 1998. Both upstream regulation of GTP/GDP exchange on G proteins and G protein-mediated

effector activation may be inhibited with interfacial binding compounds. Thus, high affinity analog peptides can be designed to specifically interfere with a particular action of one GPCR. These specifically-acting peptide analogs are useful both as pharmaceutical compounds *per se*, and as potent lead compounds in modern high throughput screens for other peptides and small molecule binders having the same specific GPCR interaction.

[0047] High throughput screening is a recent technology that has been developed primarily within the pharmaceutical industry. It has emerged in response to the profusion of new biological targets and the need of the pharmaceutical industry to generate novel drugs rapidly in a changed commercial environment. Its development has been aided by the invention of new instrumentation, by new assay procedures, and by the availability of databases that allow huge numbers of data points to be managed effectively. High throughput screening combined with combinatorial chemistry, rational design, and automation of laboratory procedures has led to a significantly accelerated drug discovery process compared to the traditional one-compound-at-a-time approach.

[0048] One critical aspect of the drug discovery process is the identification of potent lead compounds. A purely random selection of compounds for testing is unlikely to yield many active compounds against a given receptor. Typically, pharmaceutical companies screen 100,000 or more compounds per screen to identify approximately 100 potential lead compounds. On average, only one or two of these compounds actually produce lead compound series. Therefore, companies have been assaying larger and larger data sets in the search for useful compounds. Compound accessibility

then becomes an issue: historical compound collections are limited in size and availability. In contrast, large combinatorial chemistry libraries can be synthesized on demand, but at significant technical difficulty and cost. As the library sizes expand, the difficulty becomes selecting the desired compounds from these very large combinatorial libraries. When literally hundred of thousands of compounds are screened, it makes characterizing the candidate lead compounds (artificial and real) an expensive and time-consuming process.

[0049] The multi-step approach to the drug discovery process described here provides a solution to many of these problems. One embodiment of this invention takes advantage of the properties of G protein α subunit carboxyl termini to identify peptides which act as high affinity, competitive inhibitors of G protein/GPCR interactions. The method, however, can be used with any specific protein-protein, protein-small molecule, protein-nucleic acid interaction or the like. In addition, peptides based on any region of a Gα subunit, or any region of a Gβα dimer, which is involved in GPCR binding may be used in the same way. Many such GPCR binding regions are known in the art. The identification of high affinity competitors forms a first step in a screening and selection method which overcomes many of the disadvantages of high throughput screening by providing specific, high affinity lead compounds against which to test potentially useful pharmaceuticals. Because peptides selected by this method have affinity for their binding partner up to 1,000 times higher or more than the native protein, this step is one key to successfully screening and identifying useful pharmaceutical compounds.

[0050] A subsequent step of the process involves high throughput screening of candidate peptide or small molecule pharmaceutical compounds against the high affinity lead peptides identified in the first step. Because the lead peptide compounds are potent and specific binders to the desired receptor, screening assays testing for compounds which are competitive inhibitors and thus decrease binding of the peptide (which interfere with their high-affinity binding) will facilitate identification of those candidate compounds which bind with useful affinity. The high throughput screening step of the drug discovery process is thereby greatly simplified, because the number of false positive compounds, and compounds which are identified as binders but which bind only with low affinity, is reduced or virtually eliminated. Only those compounds with a high chance of success will be identified by the screen, therefore there are many fewer compounds which need to be characterized and further studied to identify useful, specific, potent pharmaceutical compounds. In addition, the method identifies a compound through binding directly to the precise site of interest, so that the mechanism of binding and the mechanism of action of the newly identified pharmaceutical compound does not have to be discovered and confirmed later.

[0051] The identified high affinity peptides also may be used to identify GPCR inverse agonists. The high affinity peptides bind the receptor and stabilize it in an active or "R*" conformation. Screens which are used to identify potent agonists seek out compounds which can compete with this binding and also stabilize the GPCR in its R* state. Inverse agonists, on the other hand, stabilize the GPCR in

an inactive or "R" state. Therefore, screens designed to detect dissociation of the high affinity peptide or a decrease in its affinity for the GPCR are used to identify inverse agonists.

[0052] Although this description provides examples relative to the interaction between a G protein coupled receptor and its cognate Gα protein, the methodology can be used to identify peptide inhibitors of most protein-protein interactions, specifically including any interaction between a GPCR and any region of a Gα or Gβγ G protein subunit. The high affinity peptides selected by this method may be used in high throughput screening to identify small molecules that can be used as modulators of a variety of specific biological process.

[0053] To produce very high affinity peptide GPCR blockers, the tertiary structure of a wild-type Gα carboxyl terminal peptide or any other GPCR binding peptide in its receptor-bound conformation may be studied, for example, using trNOESY (NMR). Dratz et al., Nature, 363:276-280, 1993. Structural data derived from these types of studies of G protein regions are combined with analysis of activity of substituted peptide analogs to define the minimal structural requirements for interaction of peptides with GPCR. The following experimental systems are examples of systems which can be used to define receptor-G protein interactions: (i) rhodopsin-transducin (Gαt) in retinal rod cells, (ii) β-adrenergic receptor-Gαs in C6 glioma cells, (iii) adenosine A1 receptor-Gα1 in Chinese hamster ovary cells, (iv) $GABA_B$ receptors-Gα1 in rat hippocampal CA1 pyramidal nevrons, (v) muscarinic M2 receptor-Gα1 in human embryonic kidney cells, and the like. Any GPCR or group of

GPCR which is convenient or desired can be used to define the interaction requirements, and skilled workers are aware of many methods to understand structure-activity relationships in receptor binding of this kind. Any of these methods are contemplated for use in these methods and may substitute for the particular methods of the exemplified embodiment.

[0054] The plasmid display method provides an efficient means of identifying specific and potent peptides that can serve as competitive inhibitors of protein-protein interactions. Using the information gleaned from structure-activity studies, a library of variant peptides encoding sequences related to a GPCR-binding region, for example the Gα subunit carboxyl terminus, for each of the classes of the Gα subtypes or Gβγ can be prepared. Exemplary native sequences upon which libraries may be based include those listed in Table III, below. This library advantageously contains peptides with computer-generated random substitutions within the sequence, and allows one to test a large number of peptide sequences at one time. Preferably, peptide sequences in each library are constructed such that approximately 50% of the amino acid residues are identical to the native GPCR binding region and the remaining amino acid residues are randomly selected from any amino acid. The peptides may range in size from about 7 to about 55 amino acid residues or from about 8 to about 50 amino acids long or from about 7 to about 70 amino acid residues or longer, preferably from about 9 to about 23 amino acid residues. Undecamer peptides are most preferred. Libraries may be constructed in which about 10% to about 90% of the amino acid residues unchanged from the native sequence;

however, about 30% to about 70% unchanged is preferred and about 50% is most preferred.

[0055] Alternatively, a synthetic peptide library can be based on any protein known to interact with a GPCR, using randomly created overlapping regions of the protein. The peptides may be about 7-70 amino acids long or about 8-50 amino acids long or preferably about 9 to about 23 amino acids long and most preferably about 11 amino acids long. Oligonucleotides encoding the peptides advantageously may be cloned to the 3' end of the *LacI* gene, with a linker sequence at the N-terminus of the peptide. The linker sequence is not mandatory for successful screening, but is generally preferred. Restriction enzyme sites may be placed at either end of the peptide coding sequence for cloning purposes. See Table I below for a schematic representation of a peptide library and an example of one peptide. Additional peptides which can be used are shown in Tables II and III, below. The oligonucleotides encoding the actual peptide sequences are synthesized with 70% of the correct base and 10% each of the remaining bases, leading to a biased peptide library with an approximately 50% chance of having the correct amino acid at any specific position along the peptide sequence. Different ratios of bases may be used to achieve the desired mutagenesis rate at any particular position in the sequence.

Table III. Exemplary Native G Protein Sequences for Library or Minigene Construction.*

Name	Sequence	SEQ ID NO:	Name	Sequence	SEQ ID NO:
hgt	IKENLKDCGLF	46	CryptoGba1	LQNALRDSGIL	76
hGi1/2	IKNNLKDCGLF	47	GA3_UST	LTNALKDSGIL	77
G05_DRO	IKNNLKQIGLF	48	GA1_KLU	IQQNLKKSGIL	78
GAF_DRO	LSENVSSMGLF	49	GA3_UST	LTNALKDSGIL	79
Gi-DRO	IKNNLKQIGLF	50	GA1_DIC	NLTLGEAGMIL	80
hGi3	IKNNLKECGLY	51	GA2_KLU	LENSLKDSGVL	81
hGO-1	IANNLRGCGLY	52	GA2_UST	ILTNNLRDIVL	82
hGO-2	IAKNLRGCGLY	53	MGs-XL	QRMHLPQYELL	83
GAK_CAV	IKNNLKECGLY	54	hGs	QRMHLRQYELL	84
G0_XEN	IAYNLRGCGLY	55	hGolf	QRMHLKGYELL	85
GA3_CAEEL	IQANLQGCGLY	56	GA1_COPCO	LQLHLRECGLL	86
GA2_CAEEL	IQSNLHKSGLY	57	GA1-SOL	RRRNLFEAGLL	87
GA1_CAEEL	LSTKLKGCGLY	58	GA2_SB	RRRNLLEAGLL	88
GAK_XEN	IKSNLMECGLY	59	GA1_SB	RRRNPLEAGLL	89
GA1_CAN	VQQNLKKSGIM	60	GA1_UST	IQVNLRDCGLL	90
hGZ	IQNNLKYIGLC	61	GA4_UST	RENLKLTGLVG	91
hG15	LARYLDEINLL	62	GA1_ORYSA	DESMRRSREGT	92
GA2_SCHPO	LQHSLKEAGMF	63	GQ1_DROME	MQNALKEFNLG	93
hG12	LQENLKDIMLQ	64	GA2_DIC	TQCVMKAGLYS	94
hG13	LHDNLKQLMLQ	65	GS-SCH	LQHSLKEAGMF	95
GAL_DRO	LQRNLNALMLQ	66	GA-SAC	ENTLKDSGVLQ	96
GA2_YST	ENTLKDSGVLQ	67	GA1-CE	IISASLKMVGV	97
hG14	LQLNLREFNLV	68	GA2-CE	NENLRSAGLHE	98
hG11	LQLNLKEYNLV	69	GA3-CE	RLIRYANNIPV	99
hGQ	LQLNLKEYNAV	70	GA4-CE	LSTKLKGCGLY	100
GQ_DROME	LQSNLKEYNLV	71	GA5-CE	IAKNLKSMGLC	101
G11_XEN	LQHNLKEYNLV	72	GA6-CE	IGRNLRGTGME	102
Gq_SPOSC	IQENLRLCGLI	73	GA7-CE	IQHTMQKVGIQ	103

Table I. Example for Construction of a Synthetic Peptide Library.

```
                                  Q  R  M  H  L  R  Q  Y  E  L  L
gaggtggt nnknnknnknnk attcgtgaaaacttaaaagattgtggtcgtttc taa ctaagtaaagc
   A          B                              C                 D       E
```

(SEQ ID NO:12) n = any amino acid; k = guanidine or thymidine; A = restriction enzyme site; B = linker sequence; C = oligonucleotide encoding peptide sequence (SEQ ID NO:13); D = stop codon; E = restriction enzyme site.

Table II. Gα Subunit Peptides and Corresponding DNA Constructs.

Gα Subunit	Sequence												SEQ ID NO:
Gt	I atc	K aag	E gag	N aac	L ctg	K aaa	D gac	C tgc	G ggc	L ctc	F ttc		14 15
Gi1/2	I ata	K aaa	N aat	N aat	L cta	K aaa	D gat	C tgt	G ggt	L ctc	F ttc		16 17
GRi1/2	N aac	G ggc	I atc	K aag	C tgc	L ctc	F ttc	N aac	D gac	K aag	L ctg		18 19
Gi3	I att	K aaa	N aac	N aac	L tta	K aag	E gaa	C tgt	G gga	L ctt	Y tat		20 21
Go2	I atc	A gcc	K aaa	N aac	L ctg	R cgg	G ggc	C tgt	G gga	L ctc	Y tac		22 23
Go1	I att	A gcc	N aac	N aac	L ctc	R cgg	G ggc	C tgc	G ggc	L ttg	Y tac		24 25
Gz	I ata	Q cag	N aac	N aat	L ctc	K aag	Y tac	I att	G ggc	L ctt	C tgc		26 27
G11	L ctg	Q cag	L ctg	N aac	L ctc	K aag	E gag	Y tac	N aac	L ctg	V gtc		28 29
Gq	L ctc	Q cag	L ttg	N aac	L ctg	K aag	E gag	Y tac	N aat	A gca	V gtc		30 31
Golf	Q cag	R cgg	M atg	H cac	L ctc	K aag	Q cag	Y tat	E gag	L ctc	L ttg		32 33
G14	L cta	Q cag	L cta	N aac	L cta	R agg	E gaa	F ttc	N aac	L ctt	V gtc		34 35
G15/16	L ctc	A gcc	R cgc	Y tac	L ctg	D gac	E gag	I atc	N aac	L ctg	L ctg		36 37
G12	L ctg	Q cag	E gag	N aac	L ctg	K aag	D gac	I atc	M atg	L ctg	Q cag		38 39
G13	L ctg	H cat	D gac	N aac	L ctc	K aag	Q cag	L ctt	M atg	L cta	Q cag		40 41
Gs	Q cag	R cgc	M atg	H cac	L ctc	R cgt	Q cag	Y tac	E gag	L ctg	L ctc		42 43
5' - gatccgccgccaccatggga-													-tgaa-3'

(SEQ ID NOS:44, 45)

Table III. Exemplary Native G Protein Sequences for Library or Minigene Construction.*

Name	Sequence	SEQ ID NO:	Name	Sequence	SEQ ID NO:
hgt	IKENLKDCGLF	46	CryptoGba1	LQNALRDSGIL	76
hGi1/2	IKNNLKDCGLF	47	GA3_UST	LTNALKDSGIL	77
G05_DRO	IKNNLKQIGLF	48	GA1_KLU	IQQNLKKSGIL	78
GAF_DRO	LSENVSSMGLF	49	GA3_UST	LTNALKDSGIL	79
Gi-DRO	IKNNLKQIGLF	50	GA1_DIC	NLTLGEAGMIL	80
hGi3	IKNNLKECGLY	51	GA2_KLU	LENSLKDSGVL	81
hGO-1	IANNLRGCGLY	52	GA2_UST	ILTNNLRDIVL	82
hGO-2	IAKNLRGCGLY	53	MGs-XL	QRMHLPQYELL	83
GAK_CAV	IKNNLKECGLY	54	hGs	QRMHLRQYELL	84
G0_XEN	IAYNLRGCGLY	55	hGolf	QRMHLKGYELL	85
GA3_CAEEL	IQANLQGCGLY	56	GA1_COPCO	LQLHLRECGLL	86
GA2_CAEEL	IQSNLHKSGLY	57	GA1-SOL	RRRNLFEAGLL	87
GA1_CAEEL	LSTKLKGCGLY	58	GA2_SB	RRRNLLEAGLL	88
GAK_XEN	IKSNLMECGLY	59	GA1_SB	RRRNPLEAGLL	89
GA1_CAN	VQQNLKKSGIM	60	GA1_UST	IQVNLRDCGLL	90
hGZ	IQNNLKYIGLC	61	GA4_UST	RENLKLTGLVG	91
hG15	LARYLDEINLL	62	GA1_ORYSA	DESMRRSREGT	92
GA2_SCHPO	LQHSLKEAGMF	63	GQ1_DROME	MQNALKEFNLG	93
hG12	LQENLKDIMLQ	64	GA2_DIC	TQCVMKAGLYS	94
hG13	LHDNLKQLMLQ	65	GS-SCH	LQHSLKEAGMF	95
GAL_DRO	LQRNLNALMLQ	66	GA-SAC	ENTLKDSGVLQ	96
GA2_YST	ENTLKDSGVLQ	67	GA1-CE	IISASLKMVGV	97
hG14	LQLNLREFNLV	68	GA2-CE	NENLRSAGLHE	98
hG11	LQLNLKEYNLV	69	GA3-CE	RLIRYANNIPV	99
hGQ	LQLNLKEYNAV	70	GA4-CE	LSTKLKGCGLY	100
GQ_DROME	LQSNLKEYNLV	71	GA5-CE	IAKNLKSMGLC	101
G11_XEN	LQHNLKEYNLV	72	GA6-CE	IGRNLRGTGME	102
Gq_SPOSC	IQENLRLCGLI	73	GA7-CE	IQHTMQKVGIQ	103
GA1_YST	IQQNLKKIGII	74	GA8-CE	IQKNLQKAGMM	104
GA1_NEUCR	IIQRNLKQLIL	75	GA5-DIC	LKNIFNTIINY	105

*For production of minigene constructs each nucleotide sequence should be constructed to encode the amino acids MG at the N-terminus of the peptide by using 5'-gatccgccgccaccatggga-(SEQ ID NO:44) and -tgaa-3' (SEQ ID NO:45).

[0056] The peptides are advantageously synthesized in a display system for convenience and efficiency of performing the binding reactions. For example, plasmid or phage display systems, as are known in the art, may be employed. While peptide display systems are preferred, any method which allows efficient contact of the peptides with a GPCR and determination of binding may be used.

[0057] A peptide display ("peptides on plasmids") library is a convenient system for use with this invention which exploits the high affinity bond between LacI and *lacO*. The "peptides on plasmids" display is preferred for use with this invention for two major reasons. The technique is easily set up in the laboratory. In addition, the fusion of the peptide at the carboxyl terminus of the presenting protein mimics the normal presentation for carboxyl terminal peptides during the screen. If amino terminal or interior peptides are being tested, the peptide may be cloned at the appropriate position to mimic native presentation.

[0058] The "peptides on plasmids" method for testing carboxyl terminal peptides generally works as follows. Persons of skill in the art will be able to modify these methods as needed to accommodate different conditions using this general description and the examples below as a guide. A library of peptides is created by degenerate PCR based on the native GPCR-binding peptide of interest and fused to the carboxyl terminus of LacI. The peptide library is expressed via a plasmid vector carrying the fusion gene. The plasmid also contains the Lac operon (*LacO*), and when *E. coli* transcribes and translates the LacI fusion protein, it binds back as a tetramer to the encoding plasmid through its *lacO*

DNA binding sequence, displaying the inserted sequences of interest on the plasmid. Following transcription and translation, variant peptides encoding different sequences related to the native peptide sequence therefore are displayed as carboxyl terminal extensions of the *lacI* gene. Thus, a stable LacI-peptide-plasmid complex is formed which can be screened for binding to receptor. Methods described in Gates et al., *J. Mol. Biol.* 255:373-386, 1996, the disclosures of which are hereby incorporated by reference, are suitable. See Examples 7 and 9 for exemplary methods.

[0059] The *E. coli* strain used to display the peptides was ARI814, which has the following genotype: Δ(*srl-recA*) *endA1 nupG lon-11 sulA1 hsdR17* Δ (*ompT-fepC*)*266* Δ*clpA319::kan* Δ*lacI lacZU118*. The strain contains the *hsdR17* allele that prevents restriction of unmodified DNA introduced by transformation or transduction. The *ompT-fepC* deletion removes the gene encoding the *OmpT* protease, which digests peptides between paired basic residues. the *lon-11* and *clpA* mutations also limit proteolysis by ATP-dependent, cytoplasmic proteases. The deletion of the *lacI* gene prevents expression of the wild-type lac repressor, which would compete with the fusion constructs for binding to the lacO sites on the plasmid. The *lacZ* mutation prevents waste of the cell's metabolic resources to make β-galactosidase in the absence of the repressor. The *endA1* mutation eliminates a nuclease that has deleterious effects on affinity purification, and the *recA* deletion prevents multimerization of plasmids through RecA-catalyzed homologous recombination. This strain was selected for its robust growth properties and high yields of immunocompetent cells. Transformation efficiencies of 2 x 10^{10} colonies per mg DNA typically were achieved. Although this strain of *E.*

coli is preferred, those of skill in the art are aware of many alternatives which are convenient for use with the methods described. Therefore, any suitable and convenient bacterial strain known in the art is contemplated for use with this invention.

[0060] The LacI-peptide fusion protein library may be released from the bacteria by gentle enzymatic digestion of the cell wall using lysozyme. After pelleting the cell debris, the lysate then can be added directly to immobilized receptor for affinity purification or used without purification. The display library of these peptides is screened to identify those peptides which bind with high affinity to a particular GPCR. In this way, it is possible to screen for and identify high affinity peptides which bind GPCR and can interfere with activation of the pre-selected specific G protein. The library can be screened against any desired GPCR. Since the combinatorial library contains peptides based on a particular Gα or Gβγ subunit, any GPCR which binds to or mediates signaling through that subunit or class of subunits can be used. Multiple libraries, based on the carboxyl terminal sequences or other regions of different G protein subunits may be constructed for screening the same or different GPCR.

[0061] To screen the plasmid display library, a G protein coupled receptor of interest advantageously may be immobilized on microtiter plates for screening by ELISA. A plasmid preparation (bacterial lysate) then may be added to the wells. This screening procedure, involving allowing the peptides displayed on the library plasmids to bind receptor, is sometimes referred to as "panning." Sequences that bind the receptor stick to the well so that non-binding sequences can be removed by a washing step. The adherent plasmids

then can be expanded and used to transform *E. coli*. The "panning" process generally is repeated 2 to 8 times. In general, however, 3 to 4 sequential screens are sufficient and preferred. In the later rounds of panning, parent peptide (wild type sequence) preferably is co-incubated with the plasmid preparation to bind receptors and serve as a competitive inhibitor. In this way, only high affinity sequences on the display library are captured by the immobilized receptor. The same competitive inhibition may advantageously be performed using a high affinity peptide or small molecule which has already been identified, rather than the native peptide. See Figure 1 for a schematic diagram generally describing the "panning" procedure and Example 7 for a specific embodiment. The selection process preferably is carried out in low salt buffers because high salt concentrations destabilize the LacI-*lacO* complex, and could lead to peptides becoming associated with the incorrect plasmid. For the same reason, the panning buffers preferably contain lactose, which causes the LacI to bind more tightly to *lacO*.

[0062] The selection process of this invention allows the identification of peptide sequences with higher and higher affinity binding with each round of panning. For example, diversity in an unpanned library may look like the sequences given in Table IV, below, *i.e.* highly randomized. After successive rounds of selection, the selected adherent peptides would look more like those given in Table V, below.

Table IV. Diversity in Unpanned Gq Library.

		SEQ. ID NO.
Native	LQLNLKEYNLV	106
clone #1	LLLQLVEHTLV	107
clone #2	HRLNLLEYCLV	108
clone #3	EQWNMNTFHMI	109
clone #4	SQVKLQKGHLV	110
clone #5	LRLLL*EYNLG	111
clone #6	RRLKVNEYKLL	112
clone #7	LQLRLREHNLV	113
clone #8	HVLNSKEYNQV	114

Table V. Selection in Panned Gα11 Library.

		SEQ ID NO.
Native	LQLNLKEYNLV	106
Round 1		
1	MKLNVSESNLV	115
2	LQTNQKEYDMD	116
3	LQLNPREDKLW	117
4	RHLDLNACNMG	118
5	LR*NDIEALLV	119
6	LVQDRQESILV	120
Round 2		
1	LQLKHKENNLM	121
2	LQVNLEEYHLV	122
3	LQFNLNDCNLV	123
4	MKLKLKEDNLV	124
5	HQLDLLEYNLG	125
6	LRLDFSEKQLV	126
Round 3		
1	LQKNLKEYNMV	127
2	LQYNLMEDYLN	128
3	LQMYLRGYNLV	129
4	LPLNPKEYSLV	130
5	MNLTLKECNLV	131
6	LQQSLIEYNLL	132

[0063] LacI is normally a tetramer and the minimum functional DNA binding species is a dimer. Thus, the peptides are displayed multivalently on the fusion protein,

leading to binding to the immobilized receptor in a cooperative fashion. This cooperative binding permits the detection of binding events of quite low intrinsic affinity. The sensitivity of the assay is an advantage in that initial hits of low affinity can be identified, but the disadvantage is that the signal in the ELISA does not necessarily correlate with the intrinsic affinity of the bound peptides.

[0064] One preferred ELISA, where signal strength is better correlated with affinity, involves fusing the sequences of interest from a population of clones in frame with the gene encoding a protein, for example maltose binding protein (MBP). Once the sequences have been transferred into the monomeric fusion protein, they can be overexpressed in *E. coli* and used as either crude lysates or purified fusion proteins for assay by an ELISA which detects the protein bound to receptor or any convenient assay. Those samples with an absorbance of at least two standard deviations above background may be considered to contain high affinity binding peptides. Any desired cut-off point may be used, however, depending on the assay parameters and the needs of the operator. The purified fusion proteins can be further tested by measuring their ability to compete for the site of binding on the receptor using native peptide, a LacI-peptide fusion protein, or heterotrimeric G protein. Use of competitive ELISA allows one to calculate IC_{50} values for the binding of individual fusion protein to the immobilized receptor.

[0065] Peptide fusion proteins can be analyzed in a competitive ELISA format using a fusion protein co-incubation to prevent the binding of lower affinity peptide fusion proteins to the GPCR. Any convenient protein which does not interfere with peptide binding may be used,

including for example, glutathione-S-transferase, green fluorescent protein, or ubiquitin, however a maltose binding protein fusion protein such as MB-Gα_t340-350K341R is preferred.

[0066] Cloning the library into pJS142 creates a BspEI restriction site near the beginning of the random coding region of the library. Conveniently, digestion with BspEI and SeaI allows the purification of a 900 base pair DNA fragment that may be subcloned into pELM3, a vector that directs the MBP fusion protein to the cytoplasm, a reducing environment. Alternatively, the fragment can be cloned into pELM15, a vector which directs the MBP fusion protein to the periplasm, an oxidizing environment. pELM3 and pELM15 are simple modifications of the pMALc2 and pMALp2 vectors, respectively, available commercially (New England Biolabs). Digestion of pELM3 with AgeI and ScaI allows efficient cloning of the BspEI-ScaI fragment from the pJS142 library. Any suitable method may be used which is convenient to achieve the desired result. Modifications of these methods are well known by those of skill in the art of molecular biology and are contemplated for use here.

[0067] Proof that the high affinity peptides competitively bind to GPCR and interfere with its recognition of G protein can be obtained using a competitive binding assay in the presence of a heterotrimeric G protein. For example, if rhodopsin is the GPCR used in the screen, heterotrimeric G protein, transducin (Gt) may be used. Gt binds rhodopsin with multiple epitopes and is membrane-bound via myristoylation of the α subunit and farnesylation of the γ subunit carboxyl terminus. Poor competition of peptide analog binding by carboxyl terminal native peptide constructs and/or heterotrimeric Gt indicates high affinity

binding of the peptide analogs. [illegible] panning, peptide synthesis and bind-gous strategy of employed for determining high affinit[illegible]ies may be any GPCR, for example the Thrombin rece[illegible]des that bind PAR4), dopamine receptors (D1, D2, D3, D4(PAR1, PAR3, receptors (V1a, V1b, V2) and histamine rece[illegible] vasopressin H3), using carboxyl terminal peptide librarie[illegible](H1, H2, subunit, for example Gαi, Gαs and Gαq. Once pe[illegible] any Gα with higher binding affinities have been elucidat[illegible] analogs can be exploited to inhibit GPCR-G protein interact[illegible] they

[0068] The peptides selected by this method, characterized by high affinity, specific blockade of a desired GPCR-mediated signaling event, may be used as therapeutic agents such as traditional pharmaceuticals or gene therapies to treat disorders which would benefit by inhibition of GPCR or used to screen additional libraries of compounds able to compete with the high affinity peptide analogs. Focused synthesis of new small molecule libraries can provide a variety of compounds structurally related to the initial lead compound which may be screened to choose optimal structures. This multi-step approach which gives high affinity inhibitory peptides in the first step, and small molecules in a subsequent step reduces the number of artificial hits by eliminating the lower affinity small molecules that would be selected and have to be assayed in a normal high throughput screening method. In addition, it focuses the search for molecules that bind to a specific desired site on the receptor, for example, that of the G protein binding/activation site, rather than screening for binding to any site on the receptor. Other advantages of this technology are that it is simple to implement, amenable

to many different classes of receptors, and capable of rapidly screening very large libraries of compounds.

[0069] Any method known in the art for selecting and synthesizing small molecule libraries for screening is contemplated for use in this invention. Small molecules to be screened are advantageously collected in the form of a combinatorial library. For example, libraries of drug-like small molecules, such as β-turn mimetic libraries and the like, may be purchased from for example ChemDiv (http://www.chemdiv.com), Pharmacopia (http://www.pcop.com or Combichem (http://www.combichemlab.com) or synthesized and are described in Tietze and Lieb, Curr. Opin. Chem. Biol. 2:363-371, 1998; Carrell et al., Chem Biol. 2:171-183, 1995; United States Patent No. 5,880,972, United States Patent No. 6,087,186 and United States Patent 6,184,223. Any of these libraries known in the art are suitable for screening, as are random libraries or individual compounds. In general, hydrophilic compounds are preferred because they are more easily soluble, more easily synthesized, and more easily compounded. Compounds having an average molecular weight of about 500 often are most useful, however, compounds outside this range, or even far outside this range also may be used. Generally, compounds having c logP scores of about 5.0 are preferred, however the methods are useful with all types of compounds. Simple filters like Lipinski's "rule of five" have predictive value and may be used to improve the quality of leads discovered by this inventive strategy by using only those small molecules which are bioavailable. See Lipinski et al., Adv. Drug Delivery Rev. 23:3-25, 1997.

[0070] Screening of the peptides or small molecules may be performed conveniently using receptors from any source.

Generally, it is convenient to purify receptor from cells and reconstitute the receptor in lipid vesicles or to use membranes isolated from insect or mammalian cells that overexpress the receptor. PAR1 and rhodopsin are convenient receptors, however any suitable receptor is contemplated for use with this invention. The receptors used for screening may be purified from a natural source or purified from cells which overexpress the receptor and reconstituted in lipid vesicles. Alternatively, membranes containing the receptor may be prepared from cells which natively express the receptor, for example Sf9 cells which express PAR1, or from cells which have been genetically engineered to express the receptor, for example mammalian or insect cells overexpressing PAR1. Initially, it is advantageous to determine the binding affinity of the peptide fusion protein or high affinity peptide against which the peptides or small molecules are screened. This allows the amount of receptor and peptide MBP peptide fusion protein or small molecule in the assay to be optimized.

[0071] Generally, it is convenient to test the libraries using a one well-one compound approach to identify compounds which compete with the peptide fusion protein or high affinity peptide for binding to the receptor. A single compound per well generally is used, at about 10 nM each or at any convenient concentration depending on the affinity of the receptor for the compounds and the peptide against which they are being tested. Compounds may be pooled for testing, however this approach requires deconvalution. Compounds may be pooled in groups of about 10 to about 50 compounds per well, or more, at about 10nM each or at any convenient concentration depending on the affinity of the receptor for the compounds being tested. Peptides desirably are screened

using a pooled approach because of the layer members of peptides which are screened in the first instance. Peptides may be screened individually as well, but preferably are screened in pools of about 10^4-10^{12} peptides per well or about 10^8-10^{10} peptide per well or most preferably about 10^9 peptides per well.

[0072] ELISA, or any other convenient assay, such as fluorescence assays or radioimmunoassay may be used to determine (1) if one or more peptides in each well reduce the amount of binding by the high affinity peptide fusion protein or high affinity peptide, or (2) if one or more peptides in each well bind to the receptor. Compounds may be tested at a series of concentrations, as well, and this generally is preferred if the affinity of the peptide or peptide fusion protein is not known. In an ELISA, wells in which the OD_{450} is half or less than half than that of control wells (no tested compounds) generally are considered "positive" and may be further studied. Any suitable cut-off point may be used, however, depending on the assay components and the goals of the assay.

[0073] Screening against the high affinity peptide analogs can be performed using the desired GPCR immobilized onto microtiter wells, biochips, or any convenient assay surface. Binding assays performed in solution also are suitable. One, several, or thousands of candidate small molecule pharmaceutical compounds can be screened for binding to the receptor in the presence or absence of a high affinity peptide analog. The assays preferably are performed in the presence of a high affinity binding peptide to ensure that only those candidate compounds which can successfully compete for binding against the high-affinity binding peptide will be captured by the receptor.

Alternatively, organic compounds or small molecules which have been identified by screening as competitively binding with a high affinity peptide analog may also be used as lead compounds in screening for further small molecule candidate compounds with even higher affinity. In either screening process, binding may be detected by any convenient method, for example by ELISA, fluorescence assays or radioimmunoassays.

[0074] By using a two-step protocol to identify compounds which block G protein signaling, high throughput screening of compounds and characterization of the selected compounds is significantly reduced in both time and cost, because only potent and strongly binding compounds are selected. The first step of identification of high affinity peptides which strongly compete with G proteins for their site of binding on G protein-coupled receptors insures this because the high affinity peptides are designed and tested for the particular desired binding specificity, ability to inhibit function within a cellular system and ability to inhibit functions *in vivo*.

[0075] Preferably, only the most strongly binding and effective peptide analogs or small molecules are used in the second or subsequent screening step. This two or multi-step protocol reduces the number of false positives and identification of compounds which bind only weakly by eliminating the lower affinity small molecules that would be detected and assayed in a conventional high throughput screening method. This method, therefore, is simple to implement, inexpensive, composed of only a few components, amenable to many different classes of receptors, and capable of rapidly screening large libraries of compounds. This method enables efficient identification of new classes of

small organic peptidomimetic molecules that function as inhibitors of receptor action, for example, thrombin receptor inhibitors, dopamine receptor inhibitors, histamine receptor inhibitors, or vasopressin receptor inhibitors. These identified compounds can target a single GPCR, a class of GPCR, or block a single G protein pathway activated by GPCR.

[0076] Thorough evaluation of the selected compounds (either peptides or small molecules) for use as therapeutic agents may proceed according to any known method. Properties of the compounds, such as pK_a, log P, size, hydrogen bonding and polarity are useful information. They may be readily measured or calculated, for example from 2D connection tables. Association/dissociation rate constants may be determined by appropriate binding experiments. Parameters such as absorption and toxicity also may be measured, as well as *in vivo* confirmation of biological activity.

[0077] Pharmaceutical preparations are prepared by formulating the peptides or small molecules identified by the inventive screen according to methods well known in the art, with any suitable pharmaceutical excipient or combination of pharmaceutical excipients. Preparations may be made for administration by any route, such as intravenous, intramuscular, subcutaneous, oral, rectal, vaginal, transdermal, transmucosal, sublingual and the like, however, the intravenous route is generally preferred for peptide preparations. Any suitable vehicle may be used, for example saline or lactated Ringer's, for intravenous administration.

[0078] Dosages for treatment of GPCR-related diseases or condition will depend on many factors such as the nature of

the disorder, the GPCR involved, the route of administration, factors relating to the general physical condition and health of the patient and the judgment of the treating physician. Persons of skill in the art are well aware of these factors and consider manipulation of dosage to obtain an optimum result to be routine. Generally, dosages for intravenous administration may vary between about 0.01 mg/kg and 1000 mg/kg, however, this range can be expanded depending on the patient's needs. Such an expanded range is considered within the scope of this invention.

[0079] Alternatively, peptides according to this invention may be provided to cells, *in vivo* or *ex vivo*, by delivery of an expression construct. Gene therapy can be performed in-vivo as a direct introduction of the genetic material. The in vivo gene transfer would introduce the oligonucleotides encoding the peptides to cells at the site they are found in the body, for example to skin cells on an arm, or to lung epithelial cells following inhalation of the gene transfer vector. Alternatively, ex-vivo gene transfer, the transfer of genes into viable cells that have been temporarily removed from the patient and are then returned following treatment (e.g. bone marrow cells) could also be employed.

[0080] Gene transfer vectors can be engineered to enter specific tissues or cells. Transductional targeting allows the gene transfer vectors to interact with specific cell surface receptors. Transductional targeting can also take advantage of the rate of cellular division by using gene transfer vectors that target rapidly dividing cells such as tumor cells. Transcriptional targeting recruits distinct cellular promoter and enhancer elements to influence transcription of the therapeutic gene. Transfection

efficiencies are also enhanced by engineering vectors with monoclonal antibodies, carbohydrate ligands, and protein ligands that help deliver genes to specific cells.

[0081] The gene transfer vectors used to produce the high affinity peptides inside cells could be viral vectors (Retrovirus, Adenovirus, Adeno-Associated Virus, Herpes Simplex Virus, or Vaccinia Virus). As an alternative, non-viral vectors may also be used, these include such methods as injection of naked DNA, or introduction of either DNA or peptides by attachment to positively charged lipids, or cationic liposomes, electroporation or ballistic DNA Injection (limited to ex-vivo applications), as well as introduction of branched peptides.

[0082] Tet-inducible retroviral vectors for the native C-terminal sequences that co-expresses GFP driven by an internal ribosomal entry site (IRES) from encephalomyocarditis virus (p-Tet-Ti-GFP) may be used. These vectors can be modified so that they encode the high affinity peptide sequences. In addition, the high affinity peptide can be driven by a sequence allowing for spatial or temporal expression. For in vitro studies, viral supernatants may be collected from a pantropic producer line such as GP-293 (Clontech) in serum-free media. Viral supernatants may be concentrated by ultracentrifugation at 4°C for 2 hr at 22,000 rpm, and the pellets resuspended in 1/100 the original volume in serum-free media with a titer of at least 10^8 i.u. (Infectious units)/ml and stored at -80°C.

[0083] Murine leukemia virus (MLV) derived retroviral vectors are commonly used vehicles for stable delivery of therapeutic genes into endothelial cells. For the retrovirus studies in vivo, high affinity peptides subcloned

into a replication-defective murine Moloney retrovirus vector which is Tet-inducible and co-expresses GFP driven by an internal ribosomal entry site (IRES) from encephalomyocarditis virus (pTet-GFP). These constructs may then be transiently transfected into producer line to generate cell-free titers of 10^6 -10-i.u/ml. If needed, a pantropic retroviral expression system (GP-293; Clontech) which utilizes VSV-G, an envelope glycoprotein from the vesicular stomatitis virus, may be utilized to overcome low transfection efficiencies. By using this innovative cell-based gene transfer method one can obtain stable, long-term, and localized gene expression of the high affinity C-terminal peptides.

[0084] To conclusively demonstrate that the compounds identified by this method can modulate G protein signaling events implicated in disease syndromes *in vivo*, antagonism of selective G protein signal transduction events may be confirmed. One method of testing the ability of compounds to compete with native G protein binding involves expressing peptides that block the receptor-G protein interface in cells bearing the receptor. Plasmid constructs that encode GPCR-binding region peptides, such as carboxyl terminal peptide sequences from the various Gα subunits (see Table VI) can be used to express them in cells *in vivo*, *ex vivo* or *in vitro*, so that the metabolic effects of selective GPCR blockade can be studied qualitatively and quantitatively. Such studies provide proof that the binding which the compounds possess is useful *in vivo* to modulate selective G protein signals.

[0085] Expression of the peptides is conveniently achieved using the minigene approach by methods such as those described in Example 23, however any suitable method

may be used. Any desired peptide sequence may be expressed using these methods. Those of skill in the art are well aware of alternative methods for construction, transfection and expression of protein and peptide constructs comprising the high affinity peptide analogs, and such methods are contemplated for use with them.

Table VI. Exemplary Sequences of C-terminal Minigene Peptides.

Peptide Name	Sequence	SEQ ID NO:
Gαi	MGIKNNLKDCGLF	133
GαiR	MGNGIKCLFNDKL	134
Gαq	MGLQLNLKEYNAV	135
Gαq**	MGLQLNLKEYNTL	136
Gα12	MGLQENLKDIMLQ	137
Gα13	MGLHDNLKQLMLQ	138

[0086] As discussed above, many receptors interact with and activate multiple G proteins. Using the minigene strategy to introduce the high affinity-binding carboxyl terminal peptides into cells, it is possible to inhibit specific G protein-coupled receptor interactions with individual G proteins, thus demonstrating the feasibility of specific G protein blockade *in vivo* with compounds identified by the inventive method. For those receptors which activate multiple G proteins, each of which activates a distinct set of signaling pathways mediating a specific set of responses (for example, the thrombin receptor), one pathway can be inhibited without substantially affecting the others.

[0087] To selectively antagonize G protein signal transduction events *in vivo* by expressing peptides that block the receptor-G protein interface, minigene plasmid

vectors were designed to express the C-terminal peptide sequence of the various Gα subunits following their transfection into mammalian cells. A control minigene vector also was created, encoding the carboxyl terminus of $G\alpha i_{1/2}$ in random order (GαiR, see Table VI). One important element necessary for the minigene approach to block intracellular signaling pathways effectively *in vivo* is expression of adequate amounts of the desired peptides. Therefore, expression of the minigene should be confirmed by a convenient method of detecting mRNA, protein or both. Any convenient method known in the art can be used.

[0088] To determine the cellular efficacy of the minigene approach for expressing GPCR binding peptides, and to show the specific inhibition of one G protein pathway in response to a given receptor activation signal without affecting others, compounds advantageously may be assayed in a system designed to exhibit a measurable cellular signaling endpoint. One example of such a system is the thrombin receptor, PAR1, in endothelial cells. This receptor activates multiple G proteins. Several signaling endpoints, including transcription analysis of induced PAR1 gene expression; biochemical analysis of effector molecules including $[Ca^{2}+]$, MAP kinase ("MAPK") activity, adenylyl cyclase activity, and inositol phosphate accumulation; as well as functional assays such as cell proliferation and endothelial permeability are available to measure specific activation or modulation of activation of different G proteins by ligand binding at this receptor. Signaling activity may be measured by any convenient method, including: measuring inositol phosphate accumulation; measuring intracellular calcium concentration levels; measuring transendothelial electrical resistance; measuring

stress fiber formation; measuring ligand binding (agonist, antagonist or inverse agonist); measuring receptor expression; measuring receptor desensitization; measuring kinase activity; measuring phosphatase activity; measuring nuclear transcription factors; measuring cell migration (chemotaxis); measuring superoxide formation; measuring nitric oxide formation; measuring cell degranulation; measuring GIRK activity; measuring actin polymerization; measuring vasoconstriction; measuring cell permeability; measuring apoptosis; measuring cell differentiation; measuring membrane association of a protein that translocates upon GPCR activation, such as protein kinase C; measuring cytosolic accumulation of a protein that translocates upon GPCR activation, such as protein kinase C; measuring cytosolic accumulation of a protein that translocates upon GPCR activation, such as src; and measuring nuclear association of a protein that translocates upon GPCR activation, such as Ran. The functional effects of Gα C-terminal minigenes in the mechanism of thrombin-induced cell retraction, as measured by the change in transendothelial electrical resistance (TEER) also can be used to measure G protein inhibition.

[0089] For example, thrombin-mediated PAR1 gene induction was inhibited in human microvascular endothelial cells (HMEC) expressing the Gαi minigene construct. Expression of the Gαq minigene construct, however, affected thrombin-mediated inositol phosphate accumulation. Expression of Gαq also specifically decreased both thrombin-induced intracellular Ca^{++} rise and thrombin-induced MAPK activity.

[0090] Thrombin activation of the Gαi mechanism in HMEC decreases cAMP levels increased in response to isoproterenol (which acts through Gαs). Assay for cAMP level increases in

response to isoproterenol alone may be compared to increases after thrombin pre-incubation in cells expressing Gαi to show that expression of the GPCR binding peptide blocks Gαi signaling.

[0091] Recent work by Gohla et al., J. Biol. Chem. 274:17901-17907, 1999, elegantly demonstrated that thrombin receptors induce stress fiber accumulation via Gα12 in an EGF receptor-independent manner. The formation of stress fiber formation appears to be Rho dependent. Both G12 and G13 have been implicated in the Rho signaling pathway. Therefore, expression of Gα12 and Gα13 GPCR-binding peptides in HMEC were used to determine whether these peptides could block the appearance of stress fibers in response to thrombin.

[0092] The extracellular signal-regulated kinase (ERK) subfamily of mitogen-activated protein kinases (MAPKs) regulates numerous cell signaling events involved in proliferation and differentiation. This forms the basis of another assay which can determine whether GPCR binding peptides can affect a specific G protein mediated pathway. Transfection of HMEC cells with minigenes encoding GPCR binding peptides along with HA-MAPK followed by immunoprecipitation of the HA-MAPK permits measurement of the effects only on cells expressing GPCR binding peptides.

[0093] Many studies have shown that the M_2 muscarinic receptor (mAChR) couples exclusively to the Gi/GO family. See Dell'Acqua et al., *J. Biol. Chem.* 268:5676-5685, 1993; Lai et al., *J. Pharm. Exp. Ther.* 258:938-944, 1991; Offermanns et al., *Mol. Pharm.* 45:890-898, 1994; Thomas et al., *J. Pharm. Exp. Ther.* 271:1042-1050, 1994. The M_2 mAChR can efficiently couple to mutant Gαq** in which the last five amino acids are substituted with the corresponding

residues from Gαi or GαO, suggesting that this receptor contains domains that are specifically recognized by the carboxyl terminus of Gαi/O subunits. See Liu et al., *Proc. Natl. Acad. Sci. USA* 92:11642-11646, 1995.

[0094] To test inhibition of G protein-coupled receptor-mediated cellular responses by carboxyl terminal Gα peptides expressed using minigene constructs, prototypical directly Gβγ activated channels (GIRK channels) regulated by a pertussis toxin-sensitive M_2 mAChR was chosen as the model. In this model, the importance of the Gα carboxyl terminus and the downstream effector system have been well established. See Krapivinsky et al., *J. Biol. Chem.* 270:29059-29062, 1995; Krapivinsky et al., *J. Biol. Chem.* 273:16946-16952, 1998; Sowell et al., *Proc. Natl. Acad. Sci. USA* 94:7921-7926, 1997. Inhibition of M_2mAChR activation of inwardly rectifying potassium currents can be tested to demonstrate inhibition of a downstream functional response following agonist stimulation of GPCR on cells transiently transfected with a Gα carboxyl terminal peptide minigene or treated with a pharmaceutical compound identified by screening against high affinity Gα peptides.

[0095] GIRK channels modulate electrical activity in many excitable cells. See Breitwiese et al., *J. Membr. Biol.* 152:1-11, 1996; Jan et al., *Curr. Opin. Cell Biol.* 9:155-160, 1997; Wickman et al., *Curr. Opin. Neurobiol.* 5:278-285, 1995. Because the channel opens as a consequence of a direct interaction with Gβγ, whole cell patch clamp recording of I_{KACh} can be used to demonstrate inhibition of a downstream functional response following agonist stimulation of GPCR on cells transiently transfected with a Gα carboxyl terminal peptide minigene or treated with a pharmaceutical compound identified by screening against high affinity Gα

peptides. Superfusion of cells expressing GIRK1/GIRK4 with their ligand, acetylcholine (ACh), activates inwardly rectifying potassium currents.

[0096] Using well-established receptor models accepted to be indicative of *in vivo* cellular results, this type of data can show that the individual G proteins activated via a given GPCR have specific roles in mediating cellular events and can be modulated in a specific fashion by ligands mimicking GPCR binding regions of individual Gα subunits. In particular, for receptors such as the thrombin receptor, which activate multiple G proteins, each of which activates a distinct set of signaling pathways mediating a specific set of responses, it is possible using the inventive methods to block one pathway while leaving all the others functional. The high affinity peptide analogs identified *in vitro* by consecutive affinity purification and competitive binding, are capable of specifically inhibiting the downstream consequences of G protein signaling.

[0097] The assays described above clearly establish the ability of compounds identified by *in vitro* competitive binding studies to interfere with a particular GPCR-G protein interaction selectively, even when the GPCR regulates multiple G proteins within the cell. Moreover, the peptides compete very effectively with the native sequence. In addition, the minigene approach described above and exemplified in the examples below allows a systematic test of the roles of other G proteins such as Gα12 and Gα13, which may be involved in the mechanism of increase of endothelial permeability, and clearly demonstrates the viability of this approach to select and identify Gα subunit modulating compounds. The peptides

therefore are suitable for use in treatment of any disorder or syndrome characterized by G protein signaling excess.

[0098] In another aspect, the invention relates to methods to identify the G proteins with which a specific orphan receptor is coupled, using the materials provided by the invention. For example, the described methods can be used to test any GPCR with a battery of Gα subunit peptides to determine which species of G protein(s) mediates the effects of the receptor. The methods described in Examples 15-18 are suitable. Those of skill in the art are capable of designing other assays, or variations and modifications using these assays as guides.

[0099] The following non-limiting examples are provided to illustrate certain aspects of this invention.

Example 1. Construction of a Peptide Library.

[0100] Construction of a biased peptide library has been described previously. Martin et al., *J. Biol. Chem.* 271:361-366, 1996; Schatz et al., *Meth. Enzymol.* 267:171-191, 1996. The vector used for library construction was pJS142 (see Figure 2). This vector had a linker sequence between the LacI and the biased undercamer peptide coding sequence, as well as restriction sites for cloning the library oligonucleotide. The oligonucleotide synthesized to encode the mutagenesis library was synthesized with 70% of the correct base and 10% of each of the other bases at each position. This mutagenesis rate leads to a biased library such that there is approximately a 50% chance that any of the 11 codons will be the appropriate amino acid and approximately a 50% chance that it will be another amino acid. In addition, a linker of four random NNK (where N denotes A, C, G or T and K denotes G or T) codons were

synthesized at the 5' end of the sequence to make a total of 15 randomized codons. Using this method, a library with greater than 10^9 independent clones per microgram of vector used in the ligation was constructed based on the carboxyl terminal sequence of Gαt (IKENLKDCGLF; SEQ ID NO:139). The nucleic acid used for creating this library was 5'-GAGGTGGTNNKNNKNNKNNKatcaaggagaacctgaaggactgcggcctcttcTAACTAAGTAAAGC-3', wherein N= A/C/G/T and K= G/T; SEQ ID NO:140).

Example 2. Sequences for the Creation of Gα Subunit Peptide Libraries.

[0101] Libraries were created using the methods of Example 1 and the sequences listed below in Table VII.

Table VI. C-Terminal Gα Subunit Peptide Library Constructs.

Gα Sub-unit	RE	Linker	Peptide Coding Region	Stop	RE	SEQ ID NO:
Gs	5-GAGGTGGT	NNKNNKNNKNNK	attcgtgaaaacttaaaagattgtggtcgtttc	TAA	CTAAGTAAAGC-3'	141
G11	5-GAGGTGGT	NNKNNKNNKNNK	ctgcagctgaacctgaaggagtacaatctggtc	TAA	CTAAGTAAAGC-3'	142
G12	5-GAGGTGGT	NNKNNKNNKNNK	ctgcaggagaacctgaaggacatcatgctgcag	TAA	CTAAGTAAAGC-3'	143
G13	5-GAGGTGGT	NNKNNKNNKNNK	ctgcatgacaacctcaagcagcttatgctacag	TAA	CTAAGTAAAGC-3'	144
G15	5-GAGGTGGT	NNKNNKNNKNNK	ctcgcccggtacctggacgagattaatctgctg	TAA	CTAAGTAAAGC-3'	145
Gz	5-GAGGTGGT	NNKNNKNNKNNK	atacagaacaatctcaagtacattggcctttgc	TAA	CTAAGTAAAGC-3'	146

Example 3. Isolation of Membranes from Insect Cells Expressing Thrombin Receptor.

[0102] Sf9 cells (2 x 10^8 cells) were cultured with 200 ml of Grace's insect cell culture medium (Life Technologies, Inc., Grand Island, NY) containing 0.1% Pluronic F-68 (Life Technologies, Inc., Grand Island, NY)), 10% fetal calf serum, and 20 µg/ml gentamicin in a 1-liter spinner flask at

27°C for 25 hours. Sf9 cells were infected with the ThR/pBluebac recombinant virus at a multiplicity of infection of 3-5, and cultured at 27°C for 4 days. The cells were harvested, washed with phosphate buffered saline, and then resuspended in 10 mM Tris-HCl, pH 7.4. Cells were then homoginized with a hand-held homoginizer set at low speed for 20 seconds. The broken cells were than sedimented at 17,000 x g for 15 minutes. The supernatant was discarded, and the pellet resuspended in a buffer consisting of 50 mM Tris-HCl, pH 7.4 and 10% glycerol. Concentration of receptor in the membrane preparation ranged from 1-10,000 pM/mg. For screening, a final concentration of 200 µg/ml was used. The thrombin receptors were tested for their ability to bind to the native Gq-C terminal peptide using a MBP-Gq fusion protein. (Figure 7).

Example 4. Isolation of Membranes from Mammalian Cells Overexpressing Thrombin Receptor.

[0103] PAR1 receptor cDNA (2.1 kb insert) was obtained by polymerase chain reaction and cloned into the mammalian expression vector pBJ5. The resulting plasmid was transfected into Chinese hamster ovary cells by the calcium phosphate coprecipitation method. The PAR1 transfected cells were grown with Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 units/mL penicillin and 100 µg/mL streptomycin. The cells were detached using PBS with 5 mM EDTA and washed twice in PBS. The pellet was either used immediately for membrane preparation or stored frozen at -20°C. Pellets were homogenized in 20 mM Tris-HCl, pH 7.5, with 5 mM EDTA and 0.5 mM PMSF, using a Dounce homogenizer (10 strokes) and sonicated for 10 seconds. Nuclear debris and intact cells were removed by

centrifugation at 3000 rpm for 10 minutes. The supernatant was sedimented at 12,000 xg for 30 minutes and the resulting pellet suspended in 25 mM Tris-HCl, pH 7.5, 25 mM $MgCl_2$, 10% sucrose, 0.5 mM PMSF, 50 µg/mL antipain, 1 µg/mL aprotinin, 40 µg/mL bestatin, 100 µg/mL chymostatin, 0.5 µg/mL leupeptin and 0.7 µg/mL pepstatin. The membranes were aliquoted and frozen at -80°C.

Example 5. Preparation of Rod Outer Segments.

[0104] Bovine rod outer segments (rhodopsin-containing membranes) were prepared from fresh retinas under dim red light as described by Arsharky et al., J. Biol.Chem. 269:19882-19887, 1994. The retinas were placed in a beaker for dissection filled with 200 mL of 30% (w/v) sucrose in isolation buffer (90 mM KCl, 30 mM NaCl, 2 mM N_gCl_2, 0.1 mM EDTA, 1 mM DTT, 50 µM phenylmethylsulfonyl fluoride, 10 mM MOPS, pH 7.5) on ice with constant moderate stirring of the solution during dissection. Following dissection, the retina solution was left in the dark for one hour on ice. The retina-sucrose solution was distributed into eight 50 mL tubes and sedimented at 3000 xg for four minutes at 4°C. The supernatant was decanted into eight fresh centrifuge tubes and placed on ice. The volumes of the tubes were filled to 1.5 cm below top with isolation buffer, then sedimented at 17,000 xg for 20 minutes ("spin 1").

[0105] The pellets were resuspended in a small volume of 30% sucrose and consolidated from eight tubes into four tubes. The tubes were filled to 1.5 cm below top with 30% sucrose, sedimented at 5000 xg for four minutes at 4°C, and the supernatant decanted into four clear tubes. These tubes were filled to 1.5 cm below top with isolation buffer and sedimented at 17,000 xg for 20 minutes at 4°C ("spin 2").

[0106] A stepwise sucrose gradient was prepared in six gradient tubes using the solutions in Table VIII, below, with a sequence from top to bottom of #2, #3, #4.

Table VIII. Sucrose Gradient Solutions.

Solution	#2 (0.84 M)	#3 (1.0 M)	#4 (1.14 M)
42% Sucrose	51.30 g	61.05 g	69.75 g
1.0 M MOPS	750 µL	750 µL	750 µL
2.0 M KCl	2250 µL	2250 µL	2250 µL
3.0 M NaCl	750 µL	750 µL	750 µL
2.0 M $MgCl_2$	75 µL	75 µL	75 µL
Total Weight	83.25 g	84.75 g	86.25 g

[0107] The pellets from "spin 1" and "spin 2" were resuspended in isolation using 1 mL 26% sucrose buffer per tube. After making a slurry, each tube was homogenized with a 1 mL pipette and the tubes consolidated. The pellet solution was carefully laid onto the sucrose gradients and were not allowed to invade the gradient layers. The gradient tubes were subjected to 24,000 xg for 30 minutes at 4°C in a swinging bucket rotor, after which the orange layer containing the membranes was collected, being careful to avoid the pellet or the dark solution near the pellet. The membranes were distributed into six 50 mL tubes and placed on ice. The tubes then were filled to 1.5 cm below top with isolation buffer and sedimented at 17,000 xg for 20 minutes at 4°C. The supernatant was discarded and the pellets resuspended in 1 mL isolation buffer containing 5 µg/mL pepstatin and 10 µg/mL E-64. This suspension was stored in a foil-wrapped 15 mL conical tube at -80°C until needed, then thawed, homogenized in EDTA buffer (10 mM Tris pH 7.5, 1 mM EDTA 1 mM DTT) and sedimented at 30,000 xg for 30

minutes. The supernatants were discarded and the pellets resuspended and sedimented again as described above. The pellets then were resuspended in urea buffer (10 mM Tris, pH 7.5 1 mM EDTA, 1 mM DTT, 7 M urea), homogenized and sedimented at 45,000 kg for 40 minutes. These pellets were resuspended and homogenized in Buffer A (200 mM NaCl, 10 mM MOPS, pH 7.5, 2 mM $MgCl_2$, 1 mM DTT, 100 µM PMSF), then sedimented at 30,000 xg for 30 minutes. The pellets each were resuspended and homogenized by pipetting in 1 mL buffer A and stored at -80°C in 100 µL aliquots in foil-covered tubes for use in assays. For screening, the receptor was added to wells at 10µg/ml. Binding assays were performed as in Example 15.

Example 6. Purification of PAR1 Thrombin Receptor from Insect Cells and Reconstitution of Receptors into Lipid Vesicles.

[0108] Sf9 cells (2 x 10^8 cells) were cultured in Grace's insect cell culture medium (Life Technologies, Inc., Grand Island, NY) containing 0.1% Pluronic F-68 (Life Technologies), 10% fetal calf serum and 20 µg/mL gentamicin in a 1 L spinner flask at 27°C for 25 hours. The cells were infected with ThR/pBluebac (recombinant virus) at a multiplicity of infection of 3-5 and cultured at 27°C for four days. The cells were harvested, washed with phosphate buffered saline containing 2.7 mM EDTA and stored at -70°C until used. the cells were resuspended in lysis buffer (2.5 mM Tris-HCl, pH 7.2, 7.5 mM NaCl, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mg/mL leupeptin, 10 mg/mL aprotinin, 50 mM NaF) and washed. All subsequent steps should be done on ice with cold buffers and centrifuge rotors at or below 4°C. The cells were homogenized for one minute at maximum speed and sedimented for 45 minutes at

30,000 xg. The pellet was resuspended in lysis buffer and the homogenation/ washing step repeated three times. The resulting pellet was resuspended in 30 mL solubilization buffer (20 mM Tris-HCl, pH 7.4, 15 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 mg/mL leupeptin, 10 mg/mL aprotinin, 50 mM NaF, 0.1% (w/v) digitonin, 0.1% (w/v) Na deoxychoate) and then homogenized for one minute. The suspension was stirred for 90 minutes at 4°C and then sedimented for 60 minutes at 30,000 xg. The supernatant was loaded onto an anti-PAR1 monoclonal antibody column equilibrated with solubilization buffer containing 0.2% digitonin. After application of the supernatant, the column was washed with 10 column volumes of 10 mM Tris-HCl buffer, pH 7.4, containing 0.2% (w/v) Na dodecyl maltoside. The receptor was eluted using 10 mM triethylamine, pH 11.8. The eluted fractions were neutralized immediately using 1 M HEPES, pH 6.4. The pooled fractions were dialyzed against 50 mM HEPES buffer, pH 7.4, containing 50% (v/v) glycerol, 0.1 M NaCl and 0.2% (w/v) Na dodecyl maltoside. Aliquots were stored at -80°C.

[0109] For preparation of lipid vesicles, 200 µL phosphatidylserine (50 mg/mL in $CHCl_3$; Matreya) was dried in a rotary evaporator for 30 minutes or using a stream of dry N_2. After addition of 200 µL buffer A (50 mM HEPES, 100 mM NaCl, 0.2% (w/v) Na dodecylmaltoside), the tube was sealed under an N_2 atmosphere and sonicated in a bath sonicator for 30 minutes. Reconstitution of receptors into lipid vesicles was performed the same day, using purified receptor prepared as in Example 5. Purified receptor stocks (200 µg/mL) were thawed on ice and 50 µL was incubated for 20 minutes at 4°C with the appropriate agonist peptide (100nM final concentration). In the case of thrombin receptor, the

agonist is thrombin receptor agonist peptide (100nM final concentration; CalbioChem). After addition of 80 µL sonicated lipids and 50 µL buffer A, the samples were mixed using a vortex machine and placed on ice for 10 minutes. The samples then were loaded onto a 1 mL Extracti-gel™ column which had been washed with 0.2% BSA and pre-equilibrated with 5 mL Buffer A without Na dodecylmaltoside. The reconstituted vesicles were eluted from the column with 2.5 mL HEK buffer.

[0110] Samples 100-200 µL) were collected for purity analysis by SDS-PAGE. The concentration for each batch generally was about 10-1000 µg/ml. For use, receptor was placed in microtiter plates at about 1-100 µg/ml. The purified, reconstituted thrombin receptors were tested for their ability to bind to the native Gq-C terminal peptide using a MBP-Gq fusion protein. (Figure 3). As a control, empty vesicles were also tested for their ability to bind to the native Gq-C terminal peptide using a MBP-Gq fusion protein.

Example 7. Identification of GPCR-Binding High Affinity Peptide Analogs (Panning).

[0111] Electrocompetent cells were produced as follows. A single colony of ARI814 bacteria was grown overnight at 37°C in 5 ml sterile SOP (20 g/L Bacto-tryptone; 10 g/L Bacto-yeast extract; 5 g/L NaCl; 2.5 g/L anhydrous K_2HPO_4; 1 g/L $Mg_2SO_4 \cdot 7H_2O$). One milliliter of this overnight growth was added to 500 ml SOP and the bacteria allowed to grow with the OD_{600} read 0.6-0.8. All further washing steps were done in the cold. The cells were placed in an ice-water bath for at least 15 minutes, then subjected to centrifugation at 4000 xg for 15 minutes at 4°C followed by

resuspension in 500 ml 10% glycerol. After sitting on ice for 30 minutes, the cells were washed twice more in 500 ml and 20 ml 10% glycerol with sedimentation as above, and finally sedimented at 5000 xg for 10 minutes at 4°C and resuspended in 1 mL 10% glycerol. Cells were quick frozen using dry ice and isopropanol in 100 µL aliquots for later use.

[0112] To transfect, aliquots (40 µL) of thawed ARI814 cells were placed into each of three chilled microcentrifuge tubes. A peptide display library based on the undecamer carboxyl terminal peptide of $G\alpha_t$ (SEQ ID NO:126) was prepared according to Example 1. Two microliters of library plasmid were added to the tubes and mixed. For the first round of "panning," 200 µl of the plasmid library was added. For subsequent rounds, three sets of transfections were performed (adherent plasmids from wells containing receptor (+); adherent plasmids from wells containing no receptor (-); and the PRE sample which was not incubated). See below. In each round of panning, less library was used (round 2:100 µl; round 3:50 µl; round 4:10 µl). After the panning was completed, the DNA for the Lac1 fusion protein is eluted. This DNA (50 µl) is used to transfect E. Coli cells by electroporation, using cold, sterile 0.1 cm electrode gap cuvettes. The cuvettes were pulsed one time using a BioRad *E. coli* Pulsar set to 1.8 kV, 25 µF capacity, time constant 4-5 mseconds, with the Pulser Controller unit at 200 mΩ. Immediately, 1 mL of SOC was added and the mixture transferred to a labeled 17 x 100 mm polystyrene tube. The tube was shaken for one hour at 37°C. Aliquots were taken from each set to plate 100 µL undiluted to 10^{-6} dilution samples on LB-Amp plates. Counts of the PRE plates indicated library diversity, while comparison of the (+) and

(-) plates indicated whether specific clones were being enriched by the panning procedure.

[0113] The remaining ~900 µL in the + receptor tube was added to a 1L flask containing 200 mL LB-AMP media, prewarmed to 37°C, and grown at 37°C, shaking until OD_{600} = 0.5. The tube of cells then were placed in an ice water bath for at least 10 minutes, and kept chilled at or below 4°C during the subsequent washing steps. The cells were sedimented at 5000 xg for six minutes, resuspended in 100 mL WTEK buffer, sedimented at 5000 xg for six minutes, resuspended in 50 mL TEK buffer, resedimented at 5000 xg for six minutes and resuspenced in 4 mL HEK buffer. The cells were divided into the cryovials and stored at -70°C. One tube was used for the next round of panning and the other saved as a backup.

[0114] The panning process is illustrated in Figure 1. For screening of the library by "panning," rhodopsin receptors prepared according to Example 5 were immobilized directly on Immulon 4 (Dynatech) microtiter wells (0.1-1 µg of protein per well) in cold 35 MM HEPES, pH 7.5, containing 0.1 mM EDTA, 50 mM KCl and 1mM dithiothrietol (HEK/DTT). After shaking for one hour at 4°C, unbound membrane fragments were washed away with HEK/DTT. The wells were blocked with 100 µl 2% BSA in HEKL (35 Mm HEPES; 0.1 mM EDTA; 50 mM KCl; 0.2 M α-lactose; pH 7.5, with 1 mM DTT). For rounds 1 and 2, BSA was used for blocking; in later rounds 1% nonfat dry milk was used. For the first round of panning, about 24 wells of a 96-well plate were used. In subsequent rounds, 8 wells with receptor and 8 wells without receptor were prepared.

[0115] The Gt library was thawed (2 mL aliquot) and mixed with 6 mL lysis buffer on ice. Lysis buffer contains 4.25

mL HE (25 MM HEPES: 0.1 mM EDTA; pH 7.5); 1 mL 50% glycerol; 750 µL 10 mg/mL protease-free BSA in HE; 10 µL 0.5M DTT; and 6.25 µL 0.2M PMSF. Freshly prepared lysozyme solution (150 µL 10 mg/mL lysozyme in cold HE) was added and the tube was gently inverted several times and incubated on ice for no more than two minutes. The extent of lysis is evidenced by an increase in viscosity that can be observed by noting the slow migration of bubbles to the top of the tube after mixing. Lysis was terminated by mixing in 2 mL 20% lactose and 250 µL 2M KCl. The tube was centrifuged immediately at 13,000 xg for 15 minutes at 4°C and the supernatant transferred to a new tube. A small aliquot of 0.1% (the PRE sample) was saved in a separate, labeled tube. The blocked rhodopsin receptor-coated plate was rinsed four times with HEKL/1% BSA and exposed to room light for less than five minutes on ice to activate the rhodopsin for light-activated rhodopsin (Table IX), or left in the dark for dark-adapted (inactive) rhodopsin (Table X). Immediately thereafter, the crude bacterial lysate from the peptide library (200 µL) was added to each well and allowed to shake gently for one hour at 4°C. For round 2, this same procedure was followed. In round 3, the amount of lysate used was reduced to 100 µL. In subsequent rounds, the lysate was diluted 1:10 in HEKL/BSA. In all rounds, 5-10 µL 200 µM native peptide was added to the wells to chase off peptides that were bound with lower affinity.

[0116] After incubation with the bacterial lysate, the wells were washed four times into cold HEKL/1% BSA. Sonicated salmon sperm DNA (200 µL 0.1 mg/mL in HEKL/1% BSA was added to each well and shaken gently for 30 minutes at 4°C. The plates were washed four times with cold HEKL and twice with cold HEK, then eluted by adding 50 µL/well 1 mM

IPTG/0.2 M KCl in HE with vigorous shaking at room temperature for 30 minutes. The eluants from each group of wells (+ or - receptor) were combined in one or more microcentrifuge tubes as necessary. The volume of the PRE sample which had been saved previously was brought up to match the volume of the eluant samples and precipitated in parallel with them. To precipitate, 1/10 volume of 5M NaCl was mixed with each of the samples, then 1 µL 20 mg/mL glycogen was mixed with the samples. An equal volume of RT isopropanol was then added and mixed thoroughly. The samples were subjected to centrifugation at 13,000 xg for 15 minutes and the supernatant aspirated. The pellet was washed with 500 µL cold 80% ethanol and again subjected to centrifugation at 13,000 xg for 10 minutes. The pellets of plasmid DNA were resuspended in sterile, double-distilled water, 200 µL for the PRE sample and 4 µL for the + or - receptor samples and stored at -20°C.

[0117] Both light-activated rhodopsin and dark-adapted rhodopsin were used to screen the library in this manner. See Tables IX and X, below. Six of the sequences obtained using light-activated rhodopsin were 100-1000 times more potent than the native sequence at binding rhodopsin and are listed in Table IX. When the Gαt library was used to pan light-activated rhodopsin, residues L344, C347 and G348 were invariant. Also, in each of the highest affinity sequences, the basic residue at position 341 (R341) was changed to a neutral residue. When the Gαt library was used to pan dark-adapted rhodopsin, the L344, C347 and G348 residues were no longer invariant (L344 present in 62.5% of sequences, C347 present in 25% of sequences, G348 present in 75% of sequences) and the residue at position 341 was usually unchanged. Thus, the conformation of the receptor in its

inactive, dark-adapted state allows it to bind to a different set of peptide analogs than the light-activated receptor. In addition, it appears that in the light-activated receptor, it is the last seven amino acids of the peptide which are most important (344-350) while the first six amino acids (340-345) are more important for dark-adapted rhodopsin binding.

Table IX. Light-Activated Rhodopsin High Affinity Sequences.

Clone No.	SEQ ID NO:	Sequence
Library Sequence	139	I R E N L K D C G L F
8	147	L L E N L R D C G M F
9	148	I Q G V L K D C G L L
10	149	I C E N L K E C G L F
18	150	M L E N L K D C G L F
23	151	V L E D L K S C G L F
24	152	M L K N L K D C G M F
3	153	L L D N I K D C G L F
4	154	I L T K L T D C G L F
6	155	L R E S L K Q C G L F
11	156	I H A S L R D C G L F
13	157	I R G S L K D C G L F
14	158	I F L N L K D C G L F
15/28	159	I R E N L E D C G L F
16	160	I I D N L K D C G L F
17	161	M R E S L K D C G L F
19	162	I R E T L K D C G L L
26	163	I L A D V I D C G L F
27	164	M C E S L K E C G L F

Table X. Dark-Adapted Rhodopsin High Affinity Sequences.

Clone No. Library Sequence	SEQ ID NO:	Sequence
	139	I R E N L K D C G L F
2	165	I R E K W K D L A L F
3	166	V R D N L K N C F L F
7	167	I G E Q I E D C G P F
17	168	I R N N L K R Y G M F
21	169	I R E N L K D L G L V
26	170	I R E N F K Y L G L W
33/37	171	S L E I L K D W G L F
41	172	I R G T L K G W G L F

Example 8. Screens of PAR1 with a Gq Peptide Library.

[0118] The methods of Example 7 were used to screen different sources of PAR1 receptor using the Gq library. Purified PAR1, reconstituted in lipid vesicles (Example 6), membranes prepared from Sf9 insect cells expressing PAR1 (Example 2) and membranes prepared from mammalian cells overexpressing PAR1 were used. The results of the screens are presented in Tables XI, XII and XIII, respectively. The peptide used as the competitor was LQLNLKEYNLV (SEQ ID NO:56).

Table XI. Reconstituted Purified Recombinant PAR1 Receptor; Screening Results

Clone		SEQ ID NO:	LQLNLKEYNLV	SEQ ID NO: 69
R2-16	*SWV	319	LQFNLNDCNLV	173
R2-17	FVNC	320	LQRNKKQYNLG	174
R2-18	EVRR	321	MKLKLKEDNLV	175
R2-20	*RVQ	322	HQLDLLEYNLG	176
R2-21	RLTR	323	LQLRYKCYNLV	177
R3-37	SR*K	324	LQQSLIEYNLL	178
R3-38	MTHS	325	VHVKLKEYNLV	179
R3-44	SGPQ	326	LQLNVKEYNLV	180
R3-46	ML*N	327	LRIYLKGYNLV	181

Table XII. PAR1 Receptor Sf9 Insect Cell Membranes; Screening Results.

Clone		SEQ ID NO:	LQLNLKEYNLV	SEQ ID NO: 2
S1-13	S*IR	328	MKLNVSESNLV	182
S1-18	RWIV	329	LQLNLKVYNLV	183
S1-23	G*GH	330	LELNLKVYNLF	184
S2-26	RSEV	331	LQLKHKENNLM	185
S2-30	CEPG	332	LHLNMAEVSLV	186
S2-36	HQMA	333	LQVNLEEYHLV	187
S3-6	VPSP	334	LQKNLKEYNMV	188
S3-8	QMPN	335	LQMYLRGYNLV	189
S3-10	MWPS	336	LKRYLKESNLV	190
S3-15	C*VE	337	MNLTLKECNLV	191

Table XIII. Mammalian (CHO) Cells Overexpressing PAR1; Screening Results.

Clone		SEQ ID NO:	LQLNLKEYNLV	SEQ ID NO: 2
C4-5	PRQL	338	LQLKRGEYILV	192
C4-19	VRPS	339	LQLNRNEYYLV	193
C5-10	SRHT	340	LRLNGKELNLV	194
C5-12	FFWV	341	CSLKLKAYNLV	195
C4-16	ORDT	342	LQMNHNEYNLV	196
C7-3	NFRN	343	PQLNLNAYNLV	197
C7-10	LPQM	344	QRLNVGEYNLV	198
C7-13	LSTN	345	LHLNLKEYNLV	199
C7-14	LSRS	346	LQQRLKEYSLV	200

Example 9. Identification of GPCR-Binding High Affinity Peptide Analogs (Panning)

The methods of Example 7 were repeated using recombinant reconstituted $ß_2$ adrenergic receptor panned with the Gs Library. Results of the panning screens and ELISA binding affinity of the selected peptides are shown in Table XIV, below.

Table XIV. β2-Adrenergic Receptor screened with Gs library.

		SEQ ID NO:	
Competitor	QRMHLRQYELL	84	ELISA
AG1	QGMQLRRFKLR	201	.435
AG20	RWLHWQYRGRG	202	.431
AG19	PRPRLLRFKIP	203	.361
AG2	QGEHLRQLQLQ	204	.330
AG4	QRLRLGPDELF	205	.291
BAR1	QRIHRRPFKFF	206	.218
AG3	QRMPLRLFEFL	207	.217
BAR2	QRVHLRQDELL	208	.197
AG11	DRMHLWRFGLL	209	.192
AG9	QRMPLRQYELL	210	.190
BAR3	QWMDLRQHELL	211	.185
AG18	QRMNLGPCGLL	212	.155
BAR20	NCMKFRSCGLF	213	.079
AG13	QRLHLRGYEFL	214	.054
BAR11	HRRHIGPFALL	215	.048
BAR8	ERLHRRLFQLH	216	.047
BAR40	PCIQLGQYESF	217	.028
BAR31	QRLRLRKYRLF	218	.026

Example 10. Identification of GPCR-Binding High Affinity Peptide Analogs (Panning).

[0119] The methods of Example 7 repeated using rhodopsin screening with a Gt library. Results of the panning screens and ELISA binding affinity of the selected peptides are shown in Table XV, below.

Table XV. Rhodopsin screened with Gt library.

Competitor	IRENLKDCGLF	SEQ ID NO: 14	ELISA
L33	IVEILEDCGLF	219	1.007
L4	MLDNLKACGLF	220	.908
L3	ILENLKDCGLF	221	.839
L14	LRENLKDCGLL	222	.833
L38	LLDILKDCGLF	223	.823
L15	VRDILKDCGLF	224	.621
L34	ILESLNECGLF	225	.603
L17	ILQNLKDCGLF	226	.600
L7	MLDNLKDCGLF	227	.525
L10	IHDRLKDCGLF	228	.506
L20	IRGSLKDCGLF	229	.423
L6	ICENLKDCGLF	230	.342
L8	IVKNLEDCGLF	231	.257
L13	ISKNLRDCGLL	232	.187
L10	IRDNLKDCGLF	233	.162

Example 11. Additional Peptide Analogs.

[0120] Chinese hamster ovary-expressed PAR1 was screened against the Gt, G12 and G13 libraries, using the competitor peptide indicated in Table XVI below. Additional peptide analogs were identified using the G11 library and LQLNLKEYNLV (SEQ ID NO:243) as competitor and screened for high affinity binding to PAR1 receptor obtained from different sources, indicated in Table XVII, below.

Table XVI. Peptides Identified with CHO EXPRESSED PAR1.

Gt library (IRENLKDCGLF; SEQ ID NO:14)	G12 library (LQENLKDIMLQ; SEQ ID NO:64)	G13 library (LQDNLKQLMLQ; SEQ ID NO:65)
IREFLTDCGLF 234	LQENLKEMMLQ 240	LQDNLRHLMLQ 248
IRLDLKDVSLF 235	LEENLKYRMLD 241	LQDKINHLMLQ 249
ICERLNDCGLC 236	LQEDLKGMTLQ 242	LQANRKLGMLQ 250
PRDNTKVRGLF 237	LQETMKDQSLQ 243	LIVKVKQLIWQ 251
FWGNLQDSGLF 238	PQVNLKSIMRQ 244	MRAKLNNLMLE 252
RRGNGKDCRHF 239	WQHKLSEVMLQ 245	LQDNLRHLIQ 253
	LKEHLMERMLQ 246	LQDNRNQLLF 254
	LLGMLEPLMEQ 247	

Table XVII. PAR1 Binding Peptides Screened using a G11 Library (LQLNLKEYNLV; SEQ ID NO: 2)

CHO EXPRESSED	SEQ ID NO:	Recomb/Reconst	SEQ ID NO:	SF9 EXPRESSED	SEQ ID NO:
LQLNVKEYNLV	255	LQLNVKEYNLV	275	LQLNLKVYNLV	289
LQLNRKNYNLV	256	LQLRVKEYKRG	276	LQLKHKENNLM	290
LQLRYKCYNLV	257	LQLRYKCYNLV	277	LQKNLKEYNMV	291
LQLDLKESNMV	258	LQIYLKGYNLV	278	LQVNLEEYHLV	292
LQLNLKKYNRV	259	LQFNLNDCNLV	279	LFLNLKEYSLV	293
LQLRVKEYKRG	260	LQRNKKQYNLG	280	LELNLKVYNLV	294
LQRNKKQYNLG	261	LQRNKNQYNLG	281	LPLNPKEYSLV	295
LQIYLKGYNLV	262	LQQSLIEYNLL	282	LPLNLIDFSLM	296
LQFNLNDCNLV	263	LRLDFSEKQLV	283	LPRNLKEYDLG	297
LQYNLKESFVV	264	LYLDLKEYCLF	284	LRLNDIEALLV	298
LQQSLIEYNLL	265	HQLDLLEYNLG	285	LVLNRIEYNLL	299
LQRDHVEYKLF	266	VQVKLKEYNLV	286	LHLNMAEVSLV	300
LVIKPKEFNLV	267	MKLKLKEDNLV	287	MNLTLKECNLV	301
IQLNLKNYNIV	268	SAKELDQYNLG	288	MKLNVSESNLV	302
HQLDLLEYNLG	269			LKRYLKESNLV	303
MQLNLKEYNLV	270			LKRKLKESNMG	304
VQVKLKEYNLV	271			LKRKVKEYNLG	305
QLLNQYVYNLV	272				
MKLKLKEDNLV	273				
WRLSLKVYNLV	274				

Example 12. Preparation of LacI Lysates.

[0121] In the last round of panning, several clones were selected from the (+) receptor plates and grown up overnight in LB-Amp media. Three hundred microliters of the overnight culture was diluted in 3 mL in LB-Amp media for "ELISA lysate culture." Another 30 µL was added to an equal volume of 50% glycerol was stored in labeled microcentrifuge tubes

at -70°C. The remaining 4.5 mL was used to make DNA using a standard miniprep protocol (Qiagen Spinprep™ kits) and sequenced using a 19 base pair reverse primer which is homologous to the vector at a site 56 basepairs downstream from the TAA stop condon that terminates the random region of the library (GAAAATCTTCTCTCATCCG; SEQ ID NO:306). The DNA was stored at -20°C. The ELISA lysate culture was allowed to shake for one hour at 37°C. Expression was induced by adding 33 µL 20% arabinose (0.2% final concentration) with shaking at 37°C for 2-3 hours. The culture then was subjected to sedimentation at 4000 xg for five minutes, the pellet resuspended in 3 mL cold WTEK buffer, resedimented at 4000 xg for five minutes and the pellet resuspended in 1 mL cold TEK buffer. After transfer to 1.5 mL microcentrifuge tubes, the pellet was sedimented at 13,000 xg for two minutes and the supernatant aspirated. The cell pellet was resuspended in 1 mL lysis buffer (42 mL HE, 5 mL 50% glycerol, 3 mL 10 mg/mL BSA in HE, 750 µL 10 mg/mL lysozyme in HE and 62.5 µL 0.2 M PMSF) and incubated on ice for one hour. One hundred ten microliters 2M KCl was added to the lysis mixture and inverted to mix, then sedimented at 13,000 xg for 15 minutes at 4°C. The clear crude lysate (about 0.9 mL supernatant) was transferred to a new tube and stored at -70°C.

Example 13. PAR1 Receptor-Specific Binding of LacI-Peptide Fusion Proteins.

[0122] The binding properties of the peptide encoded by individual clones were assayed as follows. Purified PAR1 receptor prepared from Sf9 insect cells (1-10,000 pg/mL in 50mM Tris Hcl, pH 7.4, 10% glycerol) was reconstituted in lipid vesicles according to Example 6. A serial dilution of

the membranes containing receptor ranging from 0.2 to 20,000 µg/mL (+/- receptor) was added to wells on a microtiter plate and shaken gently for one hour at 4°C. After washing, a 1:1 to 1:10,000 serial dilution of a LacI-Gq lysate prepared from the LacI-Gq clone according to the methods described in Example 12 was added to the wells, the plate was shaken gently for one hour at 4°C, and washed. Anti-LacI antibodies (Stratagene) were added (1:1000) and the plate shaken gently for one hour at 4°C. After washing, HRP-conjugated goat anti-rabbit antibodies (Kierkegaard and Perry Laboratories) were added (1:2500) and the plate shaken gently for one hour at 4°C. The plate was washed, color was developed using horseradish peroxidase, and then read in an ELISA reader at OD_{450}. The general methodology for the ELISA is illustrated in Figure 3. The results, see Figure 4, show that the LacI-Gq fusion protein binds thrombin receptor in a concentration dependent manner. The ability of the LacI-Gq fusion protein to bind the empty vesicles was significantly less than vesicles reconstituted with thrombin receptor.

Example 14. Screening in the Presence of a High Affinity Peptide.

[0123] To identify peptides having even higher affinity to light-activated rhodopsin than those identified by the panning procedure described in Example 7, a high affinity peptide was included in the library incubations in rounds three and four. Peptide 8 (LLENRDCGMF; SEQ ID NO:147) had been identified in the first screening as a peptide exhibiting binding to light-activated rhodopsin 1000-fold higher than the native sequence. Screening of the Gαt library was performed as in Example 7, except that 10 µL 100 µM (100 nM final concentration) peptide 8 was included in

the wells in rounds three and four. This screen revealed several clones that both bind rhodopsin with very high affinity and stabilize it in its active form, metarhodopsin II. See Table XVIII, below. Comparing Tables IX and XVIII, it is clear that the use of peptide 8 in the screen resulted in a change at position 341 to a neutral residue. Residues L344, C347 and G348 remained stable whether peptide 8 was included in the screen or not. Use of peptide 8 resulted in a higher incidence of isoleucine at position 340 (17% with native peptide versus 71% with peptide 8) and a lower incidence of glutamine at position 342 (67% with native peptide versus 29% with peptide 8) This type of information not only contributes to the discovery of highly potent analog peptides for use as drugs or drug screening compounds, but also furthers the understanding of the structural framework which underlies the sites of contact between Gα and receptor.

[0124] Binding assays performed on some of the clones identified in this way are shown in Figure 5. All peptides identified using peptide 8 in the screening process bound with equal or greater affinity to light-activated rhodopsin as did peptide 8. Compare the first bar (HAP=peptide 8) with the remaining bars.

Table XVIII. Exemplary Light-Activated Rhodopsin High Affinity Sequences Identified in Screens with Addition of Peptide 8.

Clone No.	SEQ ID NO:	Sequence
Library Sequence	14	I R E N L K D C G L F
Peptide 8	147	L L E N L R D C G M F
3	307	I L E N L K D C G L L
7	308	M L D N L K D C G L F
8	309	I V K N L E D C G L F
10	310	I R D N L K D C G L F
13	311	I S K N L R D C G L L
17	312	I L Q N L K D C G L F
19	313	M L D N L K A C G L F

Example 15. Subcloning into MBP Vectors and Preparation of MBP Crude Lysates.

[0125] pELM3 was digested at room temperature with AgeI (New England Biolabs) and the cut vector was separated from uncut vector on a 0.7% agarose gel. DNA was purified (Qiagen Extract-a-gel kit) and digested with ScaI (New England Biolabs). The 5.6 kb MBP vector fragment was separated on a 1% agarose gel and purified as above. During the final affinity purification round of the peptide Library, a 20 mL portion of the 200 mL amplification culture was set aside before harvesting the cells. This 20 mL portion was allowed to grow to saturation, usually overnight and DNA was prepared from the cells (Qiagen midi-prep kit). The pJS142 plasmid DNA was digested with BspEI and ScaI. The 0.9 kb peptide-encoding fragment was separated from the 3.1 and 1.7 kb vector fragments on a 1% agarose gel and purified.

[0126] Different ratios of the 5.6 kb MBP vector fragment and the peptide-encoding 0.9 kb fragment (1:2, 1:1, 2.5:1,

5:1, 10:1) were ligated in ligase buffer containing 0.4 mM ATP at 14°C overnight with T4 DNA ligase. The ligation was terminated by increasing the temperature to 65°C for ten minutes. To lower the background, the ligation mixture was digested with XbaI before isopropanol precipitation using 1 µL glycogen as a carrier. After one wash with 80% ethanol, the pellet was resuspended in 20 µL double-distilled water. ARI814 cells were transformed as described in Example 7 using 1 µL of the precipitated XbaI digested ligation mix. After allowing the cells to shake for one hour at 37°C in 1 mL SOC, 100 µL of the suspension was spread on LB-Amp Plates. Crude lysates were prepared as described for LacI lysates in Example 9.

Example 16. MBP - Peptide Fusion Protein Purification.

[0127] An overnight culture (1 mL) of a single MBP-peptide fusion protein clone was inoculated into 200 mL LB-AMP media. The culture was shaken at 37°C until OD_{600} = 0.5. Protein expression was induced by addition of 150 µL 1 M IPTG (final concentration 0.3 mM), with continued shaking at 37°C for two hours. The culture then was sedimented at 5000 xg for 20 minutes and resuspended in 5 mM column buffer (10 mM Tris, pH 7.4; 200 mM NaCl; 1 mM EDTA; 1 mM DTT) and 16.25 µL 0.2 M PMSF was added. The resuspended cell pellet was then stored at -70°C. The stored pellet was thawed in cold water and placed in an ice bath. The pellet was sonicated in short pulses of less than 15 seconds with a Fisher Scientific 55 Sonic Dismembrator (40% constant time, output 5, repeating five times with a total one minute duration). The sonicated pellet was subjected to centrifugation at 9000 xg for 30 minutes, after which the supernatant was saved and diluted to 100 mL using column buffer. Usually, the protein

concentration was approximately 2.5 mg/mL. A column was prepared by pouring 7.5 ml amylose resin in a BioRad disposable column and washing with eight volumes of column buffer. The diluted crude extract was loaded by gravity flow at about 1 mL/min and the column was washed again with eight volumes of column buffer. The fusion protein was eluted with 10 mL 10 mM maltose in column buffer and concentrated using Amicon centriplus 30™ columns, then aliquoted and stored at -70°C.

Example 17. Method for Screening Library Crude Lysates by ELISA.

[0128] Microtiter wells were coated with 0.1-1.0 µg/well rhodopsin receptor in a final volume of 100 µL HEK containing 1 mM DTT with shaking at 4°C for one hour. The wells then were blocked with bovine serum albumin (BSA) by adding 100 µL 2% BSA in HEK with 1 mM DTT to the wells and continuing shaking at 4°C for at least 30 minutes, then washed four times with HEK containing 1 mM DTT. Crude lysates were diluted 1:50 in HEK containing 1 mM DTT and added to the coated wells (100 µL per well). The plates were shaken at 4°C for one hour, washed four times with PBS/0.05% Tween™20 1 mM maltose and then probed with 100 µL 1:1000 rabbit anti-MBP antibodies (New England BioLabs) in PBS containing 0.05% Tween™ 20 and 1 mM maltose, with shaking for 30 minutes at 4°C. After another wash, the wells were probed with 100 µL 1:7500 goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase in PBS containing 1% BSA and 1 mM maltose with shaking for 30 minutes at 4°C. The plate was washed four times with PBS containing 0.05% Tween™ 20 and 1 mM maltose. Horseradish peroxidase substrate (Bio-Fx; 100 µL) was added and the

color developed for 20-30 minutes. The reaction was stopped by addition of 100 µL 2N sulfuric acid and the plate read at OD_{450}. If the color reaction occurred too quickly (less than 10 minutes) or if the background in negative control wells was too high (greater than 0.2) the assay was repeated using 1:100 or 1:200 dilutions of the crude lysates.

Example 18. Binding Assay of High Affinity Rhodopsin Binding Peptides.

[0129] The entire population of peptide-coding sequences identified in round 4 of panning (see Example 7) was transferred from pJS142 to pELM3 (New England Biolabs). This plasmid is a pMal-c2 derivative with a modified polylinker, inducible by isopropyl β-thiogalacto-pyranoside and containing the *E. coli* malE gene with a deleted leader sequence and leads to cytoplasmic expression of MBP fusion proteins. The MBP-carboxyl terminal peptide analog fusion proteins were expressed in *E. coli*.

[0130] For the assay, in the dark, 1 µg/well of ROS membranes (rhodopsin) as described in Example 5 was directly immobilized on microtiter wells in cold HEK/DTT for one hour at 4°C. The wells were rinsed, blocked with 1% BSA in HEK/DTT for one hour at 4°C and rinsed again. Bound rhodopsin was activated by exposure to light for 5 minutes on ice before addition of the MBP fusion proteins (crude bacterial lysates were diluted 1:50 in HEK with 1 µM dithiothreitol; purified proteins were used at 0.2-120 nM). The MBP-Gαt340-350K341R (pELM17) fusion protein and MBP with linker sequence only (pELM6) were present in control wells at 50nM final concentration. After 30 minutes, wells were washed and rabbit anti-MBP antibody (New England Biolabs) was added. The anti-MBP antibody was used at a 1:1000

dilution for crude lysates and a 1:3000 dilution for purified proteins. After 30 minutes, wells were rewashed and goat anti-rabbit antibody conjugated to horseradish peroxidase (1:7500 dilution for crude lysates; 1:10,000 dilution for purified proteins; Kierkagaard & Perry Laboratories) was added. After 30 minutes, the plate was washed four times with PBS containing 0.05% Tween™20. Horseradish peroxidase substrate (100 µl) was added and color was allowed to develop for about 20 minutes. The reaction was stopped by addition of 100 µl 2N sulfuric acid. The results are presented in Figure 6. Values indicate absorbance at OD_{450}. The controls for the assay was pELM 17, which encodes the MBP fusion protein $G\alpha_t$340-350K341R. pELM6, which expresses MBP protein fused to a linker sequence only, served as the negative control. "No lysate" control wells were included to reflect any intrinsic, non-specific binding within the assay. See Figure 6.

[0131] The IC_{50} values of the high affinity MBP fusion proteins ranged from 3.8 to 42 nM, up to 3 orders of magnitude more potent than the 6 µM IC_{50} of MBP-Gαt340-350K341R. In all the highest affinity sequences, position 341, which is a positively charged residue in the native sequence, was changed to a neutral residue. Leu344, Cys347, and Gly348 were found to be invariant and hydrophobic residues were always located at positions 340, 349, and 350, indicating the critical nature of these residues.

Example 19. Binding of high affinity peptides to rhodopsin can be competitively inhibited by heterotrimeric Gt.

[0132] Binding of MBP fusion proteins containing the high affinity peptide from the library (sequences from clones 8,

9, 10, 18, 23, 24, as well as pELM17 which encodes the wild-type peptide sequence, and pELM6 which contains on peptide) were assessed for their ability to bind rhodopsin (0.5 µg rhodopsin/well) in the presence or absence of heterotrimeric Gt. Lysate (50 µl) from each clone was added and incubated in the light. After 45 minutes, 1 µM heterotrimeric Gt was added and the solution incubated for 30 minutes. Anti-MBP antibody was added, followed by goat anti-rabbit alkaline phosphatase conjugated antibody and substrate. The color was allowed to develop. Absorbance data are presented in Figure 7.

Example 20. Binding of MBP Clones to PAR1

[0133] To identify high affinity peptides that bind PAR1, membranes prepared from mammalian cells (Chinese hamster ovary) overexpressing PAR1 were panned with the G11 peptide library. ELISA binding affinity results of selected clones are shown in Figure 8 for their binding to membranes prepared from SF9 cells expressing either PAR1 or the Gq-coupled muscarinic M1 receptor. To quantitate the binding, purified MBP clones were analyzed using ELISA methods in which the secondary antibody was conjugated to HRP. The binding for the control MBP-Gq fusion protein is shown. See Figure 8. The data are the average of two separate experiments done in duplicate. MBP clones PAR-13 and PAR-34 both show both high affinity binding for PAR1 as well as specificity. MBP clones PAR-23 and PAR-33 appear to be both of low affinity and low specificity. See Table XIII for the sequences.

Example 21. Binding Specificity of LacI-Peptide Fusion Proteins.

[0134] PAR1 binding clones of LacI-peptide fusion protein selected from the G11 Library were diluted 1:100 in HEK/DTT and tested for dose-responsive binding to Sf9 insect cell membranes from cells expressing no receptor, the M1 receptor (which couples to Gi) or PAR1 receptor, prepared according to Example 2. Increasing amounts of membrane as indicated in Figure 9 were coated in microtiter wells, incubated and rinsed. LacI-peptide fusion protein lysates were added, incubated and rinsed, and the receptor-bound LacI-peptide fusion protein was measured as described above using a LacI antibody. Results for a single, representative clone are presented in Figure 9, and demonstrate the specificity of the selected peptides for PAR1.

Example 22. Binding of Native Gαq-Maltose Binding Protein-Peptide Fusion Protein to PAR1.

[0135] Microtiter wells were coated with purified, reconstituted PAR1 in the presence of 100 nmoles thrombin receptor activating peptide, as described above in Example 6. Purified maltose binding protein-Gαq (MBP-Gαq) was added at the concentrations indicated in Figure 10 and incubated one hour on a shaker at 4°C. The wells were rinsed and then probed with a rabbit anti-maltose binding protein antibody, followed by alkaline phosphatase conjugated secondary antibodies, as described above. Substrate was added and the color was allowed to develop about 20 minutes. Absorbance at 405 nm was measured and dose-response curves were calculated using GraphPad Prism (version 2.0). See results in Figure 10. The calculated IC_{50} of Gαq binding to activated PAR1 was 214 nM.

Example 23. Design of Oligonucleotides for Gα Peptide Minigene Constructs.

[0136] cDNA encoding the last 11 amino acids of Gα subunits was synthesized (Great American Gene Company) with newly engineered 5'- and 3'- ends. The 5'- end contained a BamHI restriction enzyme site followed by the human ribosome-binding consensus sequence (5'- GCCGCCACC-3'; SEQ ID NO:314), a methionine codon (ATG) for translation initiation, and a glycine codon (GGA) to protect the ribosome binding site during translation and the nascent peptide against proteolytic degradation. A HindIII restriction enzyme site was synthesized at the 3' end immediately following the translational stop codon (TGA). Thus, the full-length 56 bp oligonucleotide for the $Gi\alpha_{1/2}$ carboxyl terminal sequence was 5'-gatccgccgccaccatgggaatcaagaacaacctgaaggactgcggcctcttctgaa-3' (SEQ ID NO:315) and the complimentary strand was 5'-agctttcagaagaggccgcagtccttcaggttgttcttgattcccatggtgg cggcg-3' (SEQ ID NO:316). See Figure 11. As a control, oligonucleotides encoding the $G\alpha i_{1/2}$ carboxyl terminus in random order (GαiR) with newly engineered 5'- and 3'- ends also were synthesized. The DNA was diluted in sterile ddH_2O to form a stock concentration at 100 μM. Complimentary DNA was annealed in 1X NEBuffer 3 (50 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT; New England Biolabs) at 85°C for 10 min then allowed to cool slowly to room temperature. The DNA then was subjected to 4% agarose gel electrophoresis and the annealed band was excised. DNA was purified from the band using a kit, according to the manufacture's protocol (GeneClean II Kit, Bio101). After digestion with each restriction enzyme, the pcDNA 3.1(-) plasmid vector was subjected to 0.8% agarose gel electrophoresis, the

appropriate band cut out, and the DNA purified as above (GeneClean II Kit, Bio101). The annealed/cleaned cDNA was ligated for 1 hour at room temperature into the cut/cleaned pcDNA 3.1 plasmid vector (Invitrogen) previously cut with BamHI and HindIII. For the ligation reaction, several different ratios of insert to vector cDNA (ranging from 25 µM:25 pM to 250 pM:25 pM annealed cDNA) were plated. Following the ligation reaction, the samples were heated to 65°C for 5 min to deactivate the T4 DNA ligase. The ligation mixture (1 µl) was electroporated into 50 µl competent cells as described in Example 7 and the cells immediately placed into 1 ml of SOC (Gibco). After 1 hour shaking at 37°C, 100 µl of the electroporated cells containing the minigene plasmid DNA was spread on LB/Amp plates and incubated at 37°C for 12-16 hours. To verify that insert was present, colonies were grown overnight in LB/Amp and their plasmid DNA purified (Qiagen SpinKit). The plasmid DNA was digested with NcoI (New England Biolabs, Inc.) for 1 hour at 37°C and subjected to 1.5% (3:1) agarose gel electrophoresis. Vector alone produced 3 bands. When the 56 bp annealed oligonucleotide insert is present, there is a new NcoI site resulting in a shift in the band pattern such that the digest pattern goes from three bands (3345 bp, 1352 bp, 735 bp) to four bands (3345 bp, 1011 bp, 735 bp, 380 bp). See Figure 12. DNA with the correct electrophoresis pattern was sequenced to confirm the appropriate sequence. This method may be used to insert any high affinity peptide to create a minigene constant.

Example 24. Expression of Peptides from Minigene Constructs.

[0137] Expression of the GPCR binding peptides was achieved using constructs which included minigene inserts

corresponding to the carboxyl terminal sequences of various G protein α subunits (Gαi, Gαo, Gαs, Gαq, Gα11, Gα12, Gα13, Gα14), as well as a control minigene containing the Gαi sequence in random order (GαiR). The minigene insert DNAs were made by synthesizing short complimentary oligonucleotides corresponding to the peptide sequences from the carboxyl terminus of each Gα with BamHI and HindIII restriction sites at the 5' and 3' ends, respectively. Complementary oligonucleotides were annealed and ligated into the mammalian expression vector pcDNBA3.1 according to the methods of Gilchrist et al., *J. Biol. Chem.* 274:6610-6, 1999, the disclosures of which are hereby incorporated by reference.

[0138] Human embryonic kidney (HEK) 293 cells were transfected using a standard calcium phosphate procedure according to the methods of Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harpor Laboratory Press, New York, vol. 1-3 (1989), the disclosures of which are hereby incorporated by reference. To confirm the transcription of minigene constructs in transfected cells, total RNA was isolated from the cells 48 hours post transfection with pcDNA-Gαi or pcDNA-GαiR using methods known in the art. Reverse transcriptase PCR was used to make cDNA and PCR analysis was performed using the cDNA as template with primers specific for the relevant Gα carboxyl terminal peptide insert (forward: 5'-ATCCGCCGCCACCATGGGA (SEQ ID NO:317); reverse: 5'-GCGAAAGCAGCGGGGCGCTA (SEQ ID NO:318). These primers for the Gα minigenes amplify a 434 bp fragment only if the inserted peptide-encoding oligonucleotides are present; no band is observed in cells transfected with the empty pcDNA3.1 vector. The PCR products were separated on 1.5% agarose gels. The presence

of a single 434 bp band indicated that Gα carboxyl terminus peptide minigene RNA had been transcribed. See Figure 13. Control experiments were done using a T7 forward primer with the vector reverse primer to verify the presence of the pcDNA3.1 vector, and G3DPH primers (Clonetech) to approximate the amount of total RNA.

[0139] To verify that the peptide was being produced in the transfected cells, the cells were lyzed and homogenized 48 hours post transfection according to known methods. Cytosolic extracts were analyzed by gradient reversed phase HPLC as follows: 100 µL of cytosolic fraction extract was loaded onto a C4 column (Vydac) equilibrated with 0.1% TFA in ddH_2O. The peptide was eluted using 0.1% TFA in an acetonitrile gradient which increased from 0-60% over 45 minutes. Peaks were collected, lyophilized, and analyzed using ion mass spray analysis (University of Illinois-Urbana Champagne). Mass spectrometry analysis for peak 1 from $G\alpha i_{1/2}$ peptide vector (pcDNA-Gαi) transfected cells, and from cells transfected with pcDNA-GαiR indicated that a 1450 Dalton peptide (the expected molecular weight for both 13 amino acid peptide sequences) was present in each cytosolic extract. The minigene-encoded peptides were the major peptides found in the cytosol, strongly indicating that the vectors produced the appropriate peptide sequences in large amounts.

Example 25. Interfacial G Protein Peptide Inhibition of Thrombin-Mediated Inositol Phosphate Accumulation.

[0140] HMEC were seeded onto 6-well plates 24 hours before transfection at 1 x 10^5 well. Cells were transiently

transfected with pcDNA3.1, pcDNA-Gαi, pcDNA-GαiR, or pcDNA-Gq as described in Example 21. After 24 hours, cells were incubated in 2 mL culture medium containing 4 μCi/mL [^{3}H]-myoinositol to obtain steady-state labeling of cellular inositol lipids. Transiently transfected cells were assayed for inositol phosphate (IP) accumulation 48 hours after transfection. Two hours prior to stimulation with α-thrombin, cells were washed, and medium replaced with medium containing 5 mM LiCl. Cells were stimulated with 10 nM α-thrombin for 10 minutes. Inositol phosphate (IP) formation was stopped by aspiration of the medium and addition of ice-cold methanol (final concentration 5%).

[0141] Perchloric acid-lysed cells were centrifuged at 2500 rpm, 4°C for 5 min. The supernatant containing IP was eluted through a Poly-Prep chromatography column (Bio-Rad) containing 1.6 ml anion exchange resin (DOWEX AG1-X8, formate form, 200-400 mesh). The perchloric acid-precipitated pellets (containing phosphatidylinositols and lipids) were resuspended in 1 ml chloroform-methanol-10 M HCl (200:100:1, v/v/v). These suspensions were mixed with 350 μL HCl and 350 μL chloroform and sedimented for 5 min at 2500 rpm to separate the phases. The lower, hydrophobic phase was recovered and dried in counting vials to determine the amount of radioactivity in total phosphatidylinositols. The relative amount of [3H]-IP generated was calculated as follows: ([^{3}H]-IP (cpm)/[^{3}H]-IP (cpm) + [^{3}H]-inositol (cpm)). Each value was normalized using the basal value (no thrombin stimulation) obtained in pcDNA transfected cells. See Figure 14. The results presented are the normalized mean ± SEM of at least 3 independent experiments performed in triplicate. The ** symbol indicated $p < 0.005$. Results indicate that addition of thrombin increased IP production

in control cells (pcDNA, pcDNA-GiR). Cells transfected with PcDNA-Gq had no thrombin-mediated IP production increase, while cells transfected with pcDNA-Gi had a normal response. These results indicate that the Gq C-terminal peptide can inhibit thrombin-mediated IP increases in HMEC.

Example 26. Interfacial G Protein Peptide Inhibition of Thrombin-Induced P1 Hydrolysis and Intracellular Ca^{++} Rise.

[0142] To determine whether expression of the Gαq C-terminal minigene could affect intracellular $[Ca^{++}]_i$ levels, HMEC were transfected with empty vector (pcDNA), pcDNA-Gαi, pcDNA-Gαq, or pcDNA-GαiR minigene DNA (1 µg). Transfected cells were seeded at a low confluency on coverslips in a 24-well plate 48 hours post transfection. The cells were allowed to adhere for two hours. The medium was aspirated and each coverslip was incubated with 10 µM Oregon Green 488 BAPTA-1 acetoxymethyl ester (a calcium-sensitive dye) and 0.1% (v/v) Pluronic F-127 and allowed to incubate for 20-30 minutes at 37°C, then rinsed twice with wash buffer. Basal conditions were established before addition of thrombin (~70 mM) in Ca^{++} buffer. Recordings were made every 10 seconds and continued for 170 seconds after stimulation with thrombin. Images were quantitated using NIH Image. Data from at least 70 individually recorded cells were used to calculate the changes in fluorescence (y-axis). See Figure 15A, which presents fluorescence in ($[Ca^{++}]$; level) increase 30 seconds after thrombin addition. Each bar in Figure 15A represents the mean $((F_S-F_B/F_B-1) \pm$ SEM of over 70 individually recorded cells. The ** symbol indicates $p<0.005$. Figure 15B shows the kinetics of [Ca++]; fluorescence changes after cell stimulation with thrombin. Data presented are the mean $((F_S-F_B/F_B-1) \pm$ SEM at each

recording point for cells transfected with pcDNA or pcDNA-Gαq. The arrow indicate the time thrombin was added. Each time point represents over 100 individually recorded cells.

[0143] As shown in Figure 15, following cell activation by addition of thrombin there was a transient increase in intracellular [Ca^{2+}] levels. Thirty seconds after the addition of thrombin, cells transfected with pcDNA-Gαq had a calcium response that was 44% decreased as compared to cells transfected with pcDNA (Figure 15A). pcDNA-Gαq transfected cells had a 45% decrease compared to those transfected with pcDNA when all time points measured after thrombin stimulation are averaged (Figure 15B). This decrease appears to be specific as cells transfected with pcDNA-Gαi or pcDNA-Gαir did not have any effect on thrombin stimulated intracellular [Ca^{2+}] levels. Thus, cells expressing the Gαq C-terminal peptide appear to be inhibited in their ability to stimulate intracellular [Ca^{2+}] levels following activation with thrombin, indicating a specific block of this downstream mediator by expression of Gαq.

[0144] pcDNA, pcDNA-GiR, pcDNA-Gi, pcDNA-Gq, or pcDNA-Gs minigene constructs were transfected into HMEC and used to assay inositol phosphate (IP) accumulation 48 hours later. After 24 hours, cells were reseeded onto 24-well plates and labeled with [^3H]-myoinositol (2 μCi/ml). After 48 hours, cells were rinsed, and incubated with or without thrombin (10 nM) for 10 minutes. Total IP accumulation was assayed as described above using Dowex™ columns to separate [^3H] IP. The relative amount of [^3H] IP generated was calculated as follows: ([^3H] IP (cpm)/[^3H] IP (cpm) + [^3H] inositol (cpm)). Each value was normalized by the basal value (no thrombin stimulation) obtained in pcDNA transfected cells. See Figure 16. The results presented are the normalized mean ±

SEM of at least three independent experiments performed in triplicate. The ** symbol indicated $p<0.005$.

Example 27. Prevention of Thrombin-Induced MAPK Activity by High Affinity GPCR-binding Peptides.

[0145] Hemagglutanin (HA)-MAPK (1×10^5/mL was co-transfected into HMEC with the pcDNA, pcDNA-Gαi, pcDNA-Gαq or pcDNA-GαiR minigene constructs using the methods described in Example 21. After 30 hours, cells were serum-starved for 18 hours and then treated with 10 nM thrombin for 20 minutes. Cells were then lysed with RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 10% glycerol, 10 µg/mL aprotinin and 10 µg/mL leupeptin) and HA-MAPK protein immunoprecipitated using 12CA5 antibody (Roche Molecular Biochemicals; Indianapolis, IN)(one hour, 4°C) and Protein A sepharose beads (three hours, 4°C). Immune complexes were washed three times in RIPA buffer. Kinase activity in the immunoprecipitates was measured using maltose binding protein (MBP) substrate and a kinase assay kit (Upstate Biotechnology, Inc., Lake Placid, NY). MAPK activity (nM/min/mg) was obtained for each, and the relative increase of MAPK activity (thrombin-mediated fold increase) was calculated as follows: (stimulated activity (nM/min/mg) - basal activity (nM/min/mg))/basal activity (nM/min/mg). Results are presented as the mean ± SEM of at least three independent experiments in Figure 17. A * symbol indicates $p < 0.05$.

[0146] Addition of 10 nM thrombin resulted in a 3.66 fold increase in HA-MAPK activity in cells transfected with the pcDNA control vector. Similarly, cells transfected with

pcDNA-GiR had an essentially equivalent increase in thrombin mediated MAPK activity with (4.46 fold increase). However, endothelial cells transfected with a minigene construct encoding the Gαi, Gαq, Gα12 or Gα13 GPCR binding peptides showed a significant decrease in thrombin-mediated HA-MAPK activity (59%, 57%, 50% and 77%, respectively) compared to cells transfected with pcDNA.

Example 28. Reduction of Thrombin-Induced Transendothelial Electrical Resistance.

[0147] Transendothelial electrical resistance (TEER) was measured by passing an alternating current (50 μA; 2 pulses every minute) across monolayers of HMEC expressing Gαq, Gαi, GαiR or no minigene construct. Basal TEER did not change significantly with minigene transfection. Upon addition of 10 nM thrombin, however, there was a decrease in the TEER of cells expressing the Gαq minigene compared to non-transfected cells in the presence of 10 nM thrombin. See Figure 18 (representative of multiple experiments). The decrease in transendothelial electrical resistance in response to thrombin was significantly reduced in endothelial cells transfected with the minigene for the carboxyl terminus of Gαq, while there was no effect in cells transfected with Gαi, GαiR, or empty vector. These results suggested that Gαq is partially responsible for the effects of thrombin on endothelial cell shape changes.

Example 29. Inhibition of Thrombin-Mediated Stress Fiber Formation.

[0148] HMEC cells were transfected with pcDNA, pcDNA-Gα12 or pcDNA-Gα13 minigene constructs 1 μg each/100 mm dish. As

a marker for transfected cells, the pGreenLantern-1 plasmid, containing the gene for green fluorescent protein (GFP) was co-transfected together with minigene constructs. After 48 hours, cells were serum starved for 18 hours and treated with 10 nM thrombin for 20 minutes. After exposure to thrombin, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and stained for F-actin with 1 mM rhodamine-phalloidin for 30 minutes. Cells were extensively washed, mounted using Vectashield™ antifade mounting medium (Vector Laboratories, Inc.). Cells were observed with an inverted microscope (Diaphot 200, Nikon, Inc.) equipped for both differential interference contrast microscopy and epifluorescence observation using a 60x oil-immersion objective. Fluorescence and DIC images were recorded for each cell field with a cooled, integrating CCD array camera (Imagepoint, Photometrix, Ltd.) connected to the microscope. See Figure 19 for fluorescence images showing inhibition of thrombin-mediated stress fiber formation by Gα12 and Gα13 peptides.

[0149] Serum starved cells transfected with pcDNA exhibited a thin cortical F-actin rim at their margins, and contained few stress fibers (Figure 19, panel A). Those present were inconspicious and in apparently random orientation. For HMEC transfected with pcDNA after a 20-minute exposure to thrombin actin had reorganized into prominent stress fibers, typically arranged in a parallel pattern along the longitudinal axis of the cell (Figure 19, panel B). A very different pattern was observed for cells transfected with pcDNA-Gα12 (Figure 19, panel C) or pcDNA-Gα13 (Figure 19, panel D) minigenes after exposure to thrombin. In both pcDNA-Gα12 and pcDNA-Gα13 transfected cells, thrombin stimulation did not result in the appearance

of stress fibers. In cells transfected with pcDNA-Gα13, the peripheral actin rim appears thicker and more linear, providing a clear outline of cell-cell junctions. Thus, in agreement with earlier reports, thrombin induced rapid stress fiber formation in endothelial cells. Transfection of either pcDNA-Gα12 or pcDNA-Gα13 minigenes resulted in cells that no longer showed thrombin-induced stress fiber formation. Given that stress fiber formation is dependent on the small GTPase Rho, these results concur with other findings that Gα12 and Gα13 are intimately linked to Rho signaling and demonstrates the ability of GPCR binding peptides to specifically block this G protein pathway when expressed intracellularly.

Example 30. Inhibition of G Protein Activity by GPCR Binding Peptides in Single Intact Cells.

[0150] Human embryonic kidney (HEK) 293 cells, which stably express the M_2 mACR (~400 fmol receptor/mg protein), were grown in DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco), streptomycin/penicillin (100 U each; Gibco) and G418 (500 mg/L; Gibco). Cells were grown under 10% CO_2 at 37°C. In all transfections for electrophysiological studies, the CD8 reporter gene system was used to visualize transfected cells using Dynabeads™ coated with anti-CD8-antibodies (Dynal). The following amounts of cDNA were used to transfect the cells: pC1-GIRK1 (rat)- 1 μg; πH3-CD* (human) - 1 μg; pcDNA3.1, pcDNA-Gαi, pcDNA-GαiR, pcDNA-Gαq, or pcDNA-Gαs - 4 μg. Thus, typically the total amount of cDNA used for transfecting one 10 cm disk was 7 μg. The cDNAs for GIRK1 and GIRK4 were gifts from F. Lesage and M. Lazdunski (Nice, France). A standard calcium phosphate procedure was used for transient transfection of HEK cells according to the methods of

Schenborn et al., *Meth. Mol. Biol.* 130:135-145, 2000. All assays were performed 48-72 hours post transfection.

[0151] Whole cell currents were recorded from stably M_2 mAChR-expressing HEK 293 cells that had been transiently transfected with cDNA for GIRK1, GIRK4 and either pcDNA-Gαi, pcDNA-Gαs, or pcDNA-Gαq. For the measurement of inwardly rectifying K^+ currents, whole cell currents were recorded using an extracellular solution contained 120 mM NaCl; 20 mM KCl; 2 mM $CaCl_2$; 1 mM $MgCl_2$; and 10 mM Hepes-NaOH, pH 7.4. The solution for filling the patch pipettes was composed of 100 mM potassium glutamate; 40 mM KCl; 5 mM MgATP; 10 mM Hepes-KOH, pH 7.4; 5 mM NaCl; 2 mM EGTA; 1 mM $MgCl_2$; and 0.01 mM GTP. Membrane currents were recorded under voltage clamp, using conventional whole cell patch techniques. See Bunemann et al., J. *Physiol.* 489:701-777, 1995 and Bunemann et al., *J. Physiol.* 482:81-89, 1995, the disclosures of which are hereby incorporated by reference. To minimize variations due to different transfections or culture conditions, control experiments (transfection with pcDNA-GαiR) were done in parallel. Patch-pipettes were fabricated from borosilicate glass capillaries, (GF-150-10, Warner Instrument Corp.) using a horizontal puller (P-95 Fleming & Poulsen). The DC resistance of the filled pipettes ranged from 3-6 MΩ.

[0152] Membrane currents were recorded using a patch-clamp amplifier (Axopatch 200, Axon Instruments). Signals are analog filtered using a lowpass Bessel filter (1-3 kHz corner frequency). Data were digitally stored using an IBM compatible PC equipped with a hardware/software package (ISO2 by MFK, Frankfurt/Main, Germany) for voltage control, data acquisition and data evaluation. To measure K^+ currents in the inward direction, the potassium equilibrium

potential was set to about -50 mV and the holding potential was -90 mV. Agonist-induced currents were evoked by application of acetylcholine (ACh; 1 μM) using a solenoid operated superfusion device which allowed for solution exchange within 300 msec. Linear voltage ramps (from -120 mV to +60 mV within 500 ms) were applied every 10 sec. By subtracting non-agonist dependent currents, the current voltage properties of the agonist induced currents could be resolved. To exclude experiments in which currents were recorded from cells that may not have expressed the functional channel, only those cells that exhibited a basal non-agonist dependent Ba^{2+} (200 μM) sensitive inwardly rectifying current were used for analysis. For analysis of the data, the maximal current density (peak amplitude) of ACh-induced inwardly rectifying K^+ currents was measured at -80 mV and compared.

[0153] Superfusion of HEK 293 cells transiently transfected with GIRK1/GIRK4 and either pcDNA-G_i or pcDNA-G_iR DNA with 1 μM ACh revealed that cells transfected with pcDNA-$G\alpha_i$ DNA have a dramatically impaired response to the M_2 mAChR agonist. See Figure 20, which summarizes data showing the maximum amplitude of ACh evoked currents for the different transfection conditions. The maximum current evoked by ACh was 3.7 +/- 1.5 pA/pF (n=14) in cells transfected with pcDNA-G_i, compared to 24.1 +/- 8.8 pA/pF (n=11) in cells transfected with pcDNA-G_iR. This indicates that the Gαi minigene construct completely blocked the agonist mediated M_2 mAChR GIRK1/GIRK4 response while the control minigene construct (pcDNA-GiR) had no effect. Compare Figure 20A to Figures 20B and 20C. Cells transfected with minigene constructs encoding Gα carboxyl termini for Gαq or Gαs pcDNA-Gαq or pCDNA-Gαs (Figure 20)

were not significantly different than those of cells transfected with the control vectors. These findings confirm the specificity of the inhibition of M_2 mAChR-activated G protein-coupled inwardly rectifying K^+ current responses by expression of the Gαi minigene.

Example 31. Selective G Protein Signaling Inhibition in Human Microvascular Endothelial Cells.

[0154] Different measures of G-protein signaling final actions were assayed in human microvascular endothelial cells (HMEC) which natively express the thrombin receptor, PAR1. The cells were seeded onto 6-well plates at 1 X 10^5 cells/well and transiently transfected after 24 hours with minigene constructs containing Gα carboxyl terminal peptides (pcDNA, pcDNA-Gαi, or pcDNA-GαiR; 1 μg per well) using Effectene (Qiagen) according to the manufacturer's protocol. After 24 hours, the cells were labeled with 3 μCi/ml [^{3}H]-adenine for 30 minutes at 37°C. After another 24 hours, the cells were washed with serum-free medium containing 1 mM isobutyl-methyl xantine. To stimulate cAMP accumulation, cells were treated with 1 μM isoproterenol for 30 minutes at 37°C. To see the inhibitory effects of thrombin on cAMP accumulation, cells were pretreated with thrombin (50 nM) for 15 minutes prior to addition of isoproterenol. The reactions were terminated by aspiration of media followed by addition of ice-cold 5% trichloroacetic acid.

[0155] Results are provided in Figure 21 as (cAMP/cAMP + ATP) X 1000. Three separate experiments were done in duplicate. The ** symbol indicates $p<0.005$. Basal cAMP levels were essentially equivalent for all conditions tested. Endothelial cells stimulated with isoproterenol to activate β-adrenergic receptors increase their cAMP levels

through the Gs pathway. Cells transfected with pcDNA, pcDNA-Gαi, or pcDNA-GαiR showed little difference with 82, 64, and 77 fold increases in isoproterenol-mediated cAMP accumulation, respectively. When the endothelial cells were pre-incubated with thrombin prior to addition of isoproterenol, a decrease in cAMP levels was observed due to thrombin activation of the Gi pathway. Endothelial cells transfected with pcDNA and pre-incubated with thrombin showed a 39% decrease in cAMP level over cells stimulated with only isoproterenol. Similarly, cells transfected with pcDNA-GαiR and pre-incubated with thrombin showed had a 43% decrease over cells stimulated with only isoproterenol. However, cells transfected with pcDNA-Gαi and pre-incubated with thrombin had only a 0.1% decrease in cAMP levels as compared to cells stimulated with only isoproterenol. Thus, cells expressing the Gαi C-terminal peptide appear to be unable to inhibit adenyl cyclase following activation with thrombin, indicating that thrombin-mediated Gi signaling was specifically blocked by expression of the pcDNA-Gαi minigene.

Example 32. Screening Method to Identify Inverse Agonists.

[0156] Urea-washed rod outer segment membrane fragments containing rhodopsin receptor are immobilized onto microtiter wells and blocked as described in Example 7. The receptor is light-activated. Labeled native Gαt carboxyl terminal peptide is added to each well and allowed to shake gently for one hour at 4°C. The wells are washed to remove unbound peptide. Crude bacterial lysates (labeled) from a Gαt carboxyl terminal peptide prepared according to the methods described in Example 7 (200 µL) are added to each well and incubated with shaking for one hour at 4°C.

[0157] The wells then are washed to remove unbound label. The supernatants or well-bound labels are quantitated by ELISA to detect dissociation of labeled native peptide from the receptor after incubation with library peptides compared to control well incubated in the absence of library peptides.

Example 33. Small Molecule Library Screening Method.

[0158] Small molecule libraries are screened for inhibition of GPCR-mediated G protein signaling as follows. PAR1 thrombin receptor prepared from insect cells according to Example 2 are immobilized onto microtiter wells, blocked and washed according to the methods described in Example 14. A small molecule library purchased from Chem Div (San Diego, CA) are added simultaneously with MBP-peptide fusion protein (0.1-1000 nM) in a 96- or 384- well plate and allowed to shake for one hour at 4°C. Initial screens are performed with the small molecules at about 1-1000 pM. The wells are washed four times in cold PBS containing 0.05% Tween 20™ and 1 mM maltose. The amount of maltose binding protein adhering to the wells is quantitated with anti-MBP antibodies as described in Example 14, versus control wells incubated without library compounds.